



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Daniel E. H. AFAR, et al.

Serial No.: 09/455,486

Filing Date: 6 December 1999

For: Novel Serpentine Transmembrane
Antigens Expressed in Human Cancers
and Uses Thereof

Examiner: Gary B. Nickol, Ph.D.

Group Art Unit: 1642

DECLARATION OF KAREN JANE MEYRICK MORRISON, PH.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Karen Jane Meyrick Morrison, declare as follows:

1. I have a Ph.D. in Pathology from The University of Southampton, U.K. I have worked in the field of histopathology and immunohistochemistry for nearly 25 years. A copy of my curriculum vitae is attached as Exhibit A.
2. I hold the position of Research Scientist at Agensys, Inc., and I run the tissue analysis facility at Agensys. I carry out all procedures associated with histology, including the preparation, processing, cutting, staining and analysis of samples by histological, histochemical and immunohistochemical techniques. These activities include analyses of tissues and cells by bright field microscopy, fluorescence microscopy and computer-aided systems.

OK
to enter
09/25/03

3. I have reviewed the specification and claims of the above captioned application. I note that the claims are directed to STEAP-2 polypeptides. Various uses of STEAP-2 polypeptides, as well as uses predicated on immunological responses related to STEAP-2, are clearly available to those in this field based on the disclosure of the current application and knowledge available at the time this case was filed, as I will discuss below.

4. The use of STEAP-2 polypeptide for diagnostic purposes is disclosed throughout the application. For example, without limitation, at page 17 lines 1–11; page 20, lines 11–22; page 24, lines 30–35; page 26, lines 19–28; page 29, lines 1–7; and, page 32, line 36 through page 33, line 9.

5. In addition, the application provides various disclosures regarding the use of immunohistochemical techniques. In particular, immunohistochemistry is disclosed in the application as-filed, for example, at page 25, lines 9–12, and 23–27; page 29, lines 1–7; page 41, line 1 through page 42, line 22.

6. Therefore, the application explicitly discloses the use of STEAP-2 as a diagnostic target, as noted in paragraph 4 above. Accordingly, one can evaluate levels of STEAP-2 and have a useful indication of whether that tissue is malignant. In certain cases such as prostate or breast the level of STEAP-2 increases above normal and serves as an indication of malignancy. In other cases, normal and malignant tissue both express STEAP-2 at comparable levels; this is true for tissues such as colon or bladder; this is also true for tissues such as prostate or breast, mentioned above, in the course of increasing during malignant pathology. This last point is significant as it is the basis for useful diagnostic application of the STEAP-2 protein in conditions other than advanced disease.

7. In situations where STEAP-2 is present in malignant tissue, one of skill in the field of histological assessment can perform a variety of analyses such as staining for the STEAP-2 protein. In addition, when STEAP-2 is present in both normal and malignant tissue, one can evaluate any alteration of subcellular localization of the STEAP-2 protein relative to normal.

8. For example, by use of any number of well known histological methods, such as discussed in paragraph 5 above, conclude that a biopsied tissue is, e.g., malignant and that the patient from whom the biopsy was obtained has cancer. This is a vitally important diagnostic and/or prognostic outcome from a pathology evaluation in a medical or scientific setting. Other meaningful diagnoses based, e.g., on cellular characteristics such as dysplasia or neoplasia are also possible. A histopathology diagnosis need not be of cancer to be important.

9. The claimed polypeptide and immunogenic compositions thereto are also useful in view of the phenomena of altered subcellular protein localization in disease states. This is a level of information not available from routine tissue staining. Alteration of cells from a normal to a diseased state is correlated with changes in cellular morphology and is often associated with changes in subcellular protein localization/distribution. For example, cell membrane proteins that are expressed in a polarized manner in normal cells can be altered in disease, resulting in distribution of the protein in a non-polar manner over the whole cell surface. The ability to make such diagnostic decisions on the basis of altered subcellular localization was well known to persons of ordinary skill in the histology and pathology arts at the time the present application was filed.

10. The phenomenon of altered subcellular protein localization in a disease state has been demonstrated, e.g., with MUC1 and Her2 protein expression by use of

immunohistochemical means. Normal epithelial cells have a typical apical distribution of MUC1, in addition to some supranuclear localization of the glycoprotein, whereas malignant lesions often demonstrate an apolar staining pattern (Diaz et al, 2001, The Breast Journal, 7; 40-45 (Exhibit B); Zhang et al, 1998, Clinical Cancer Research, 4; 2669-2676:(Exhibit C); Cao et al, 1997, The Journal of Histochemistry and Cytochemistry, 45; 1547-1557 (Exhibit D)).

11. In addition, the phenomenon of altered subcellular protein localization in a disease state has been demonstrated, e.g., with HER2. Normal breast epithelium is either negative for Her2 protein or exhibits only a basolateral distribution whereas malignant cells can express the protein over the whole cell surface (De Potter et al, 1989, International Journal of Cancer, 44; 969-974 (Exhibit E); McCormick et al, 2002, American J. Clinical Pathology, 117: 935-943 (Exhibit F)). Alternatively, distribution of the protein may be altered from a surface only localization to include diffuse cytoplasmic expression in the diseased state. Such an example can be seen with MUC1 (Diaz et al, 2001, The Breast Journal, 7; 40-45 (Exhibit G)).

12. Alteration in the localization/distribution of a protein in the cell, as detected by immunohistochemical methods, can provide valuable information concerning the favorability of certain treatment modalities. This last point is illustrated by a situation where a protein may be intracellular in normal tissue, but cell surface in malignant cells; the cell surface location makes the cells favorably amenable to antibody-based diagnostic and treatment regimens. Accordingly, the ability to determine whether alteration of subcellular protein localization occurred for STEAP-2 makes the claimed STEAP-2 protein very useful. Use of the claimed compositions allows practitioners to make important diagnostic and therapeutic decisions.

13. Moreover, the ability to make diagnostic decisions, such as described herein, was well known to persons of ordinary skill in the histology and pathology arts at the time the present

application was filed. Also well known to such persons was an appreciation of the value of such findings in choosing amongst various treatment approaches.

14. It is important to note that a histopathology diagnosis is made from a biopsy obtained from a specific tissue site or organ. The specific tissue site or organ is disclosed to the scientist in the routine course of requesting an assessment. This is quite different than an artificial situation where a histology or binding event is asserted to be useful as "tissue typing." As I understand this concept of tissue typing, an asserted use is simply information that one tissue has the protein and another tissue lacks the protein. Apparently this fact alone is asserted to give the protein useful meaning.¹

15. Immunohistochemical reagents specific to STEAP-2 are also useful to detect metastases of tumors expressing STEAP-2 when the polypeptide appears in tissues where STEAP-2 is not normally produced. As shown in Figures 14 and 15, expression is substantially absent in many tissues and the presence of the polypeptide in these tissues in a subject, e.g., a subject diagnosed with a tumor that expresses STEAP-2, is evidence of metastasis in that individual.

16. In summary, claimed STEAP-2 polypeptides and antibodies resulting from immune responses thereto are useful in a variety of important contexts, uses supported by the specification as-filed.

17. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these

¹ Of note, however, a tissue-related conclusion can be important when evaluating a tissue for the possibility of metastases. For example, if an organ does not express a protein, but that organ is a site of metastasis for a particular cancer that does express the protein, the existence of the protein in that organ can indicate the presence of metastases there.

statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Santa Monica, California on 25 July 2003.



Karen Jane Meyrick Morrison, Ph.D.

(A)

MARY FARIS, PH.D.

ADDRESS: 2538 Almaden Court Agensys Inc.
Los Angeles, CA 90077 1545 17th Street
310-859-8508 Santa Monica, CA 90404
310-820-8029

EDUCATION:

- 1986: B.S. with Distinction,
 University of the State of New York
- 1991: Ph.D. in Immunology and Microbiology
 The Ohio State University
 Dissertation Title: Characterization of the Mechanism of Persistent I-A
 Expression by Macrophages.
 Advisor: Dr. Bruce S. Zwillling
- 1991-1994: Postdoctoral Fellowship, University of Virginia.
- 1994-1996: Postdoctoral Fellowship, UCLA School of Medicine.

EXPERIENCE:

- 1987-1989: Teaching Assistant, The Ohio State University.
 Microbiology in Relation to Man (Micro 509).
 General Microbiology (Micro 520).
 Principles of Infection and Immunity (Micro 522).
 Cellular Aspects of the Immune System (Micro 632).
- 1989-1991: Graduate Research Associate, The Ohio State University.
 Sponsor: Dr. B. Zwillling
 Research Interests: Regulation of MHC class II Expression by IFN γ -
 mediated Pathways.
- 1991-1994: Postdoctoral Fellow, University of Virginia.
 Sponsor: Dr. S. M. Fu.
 Research Interests: CD40-Mediated Signal Transduction in lymphocytes.
- 1994-1996: Postdoctoral Fellow, UCLA School of Medicine.
 Sponsor: Dr. A. Nel.
 Research Interests: Signaling Pathways Mediating T cell Activation.
 Signaling Pathways Involved in the Growth of Kaposi's Sarcoma tumors.
- 1996-1998: Researcher, Faculty, UCLA School of Medicine.
 Research Interests: Mechanism of T cell Activation and Apoptosis.
 Integration of multiple factors in the angiogenesis/tumorigenesis of
 Kaposi's sarcoma lesions.

1998-1999: Senior Scientist, Incyte Genomics.

Research Interests: Mechanism of Tumor Growth and Progression.
Integrated approach to Cancer Biology and Therapeutic Intervention

1999-present: Research Scientist III, Group Leader, Agensys Inc.

Research Interests: Functional Validation of Novel and Recently Discovered Genes
as Therapeutic Targets for Treatment of Cancer

PUBLICATIONS:

1. Faris M. and B.S. Zwilling. Somatic Cell Hybrids between Macrophages from Bcg^r and Bcg^s Mice: Characterization of MHC Class II Expression. Cellular Immunology, 1990, 127:120.
2. Faris M. and B.S. Zwilling. Characterization of the Persistent I-A Expression by Macrophages from Bcg^r Mice. J. Leuk. Biol., 1990, 49:289.
3. Zwilling B.S., M. Dinkins, R. Christner, M. Faris, A. Griffin, M. Hillberger, M. McPeck and D. Pearl. Restraint Stress Induced Suppression of MHC Class II Expression by Murine Peritoneal Macrophages. J. Neuroimmunology, 1990, 29:125.
4. Zwilling B.S., D. Brown, R. Christner, M. Faris, M. Hillberger, C. Van Epps and B.A. Hartaub. Differential Effect of Restraint Stress on MHC Class II Expression by Murine Peritoneal Macrophages. Brain Behavior and Immunity, 1990, 4:330.
5. Faris M. and B.S. Zwilling. Characterization of the Induction of Persistent MHC class II Expression by Hybrids of Macrophages from Bcg^r and Bcg^s Mice. Euro. J. Immunol., 1991, 21:1047.
6. Faris M., F. Gaskin, R.S. Geha and S.M. Fu. Tyrosine Phosphorylation Defines a Unique Transduction Pathway in Human B Cells Mediated via CD40. Trans. Assoc. Amer. Phys. 1993, 106:187.
7. Brown D., M. Faris, M. Hilburger and B.S. Zwilling. The Induction of Persistent I-A Expression by Macrophages from Bcg^r Mice Occurs via a Protein Kinase C Dependent Pathway. J. Immunol., 1994, 152:1323.
8. Faris M., F. Gaskin, J.T. Parsons and S.M. Fu. CD40 Signaling Pathway: Anti-CD40 mAb Induces Rapid Dephosphorylation and Phosphorylation of Tyrosine-Phosphorylated Proteins Including Protein Tyrosine Kinase Lyn, Fyn and Syk and the Appearance of a 28kD Tyrosine Phosphorylated Protein. J. Exp. Med., 1994, 179:1923.
9. Faris, M., B. Ensoli, N. Stahl, G. Yancopoulos, A. Nguyen, S. Wang and A. Nel. Differential Activation of the ERK, JNK and JAK-Stat Pathways by Oncostatin M and Basic Fibroblast Growth Factor in AIDS-Related Kaposi's Sarcoma cells. AIDS, 1996, 10:370.
10. Faris, M., N. Kokot, L. Lee and A.E. Nel. Regulation of IL-2 transcription by inducible

stable expression of dominant negative and dominant active MEKK-1 in Jurkat T cells. Evidence for the importance of Ras in a pathway which is controlled by dual receptor stimulation. *J. Biol. Chem.*, 1996, 271:27366.

11. Faris, M., N. Kokot, N. Stahl and A.E. Nel. Involvement of Stat3 in Interleukin-6-induced IgM production in a human B cell line. *Immunol.*, 1997, 90:350.
12. Faris, M., B. Ensoli, N. Kokot and A.E. Nel. Inflammatory Cytokines Induce AP-1 Response Elements: Activation of the bFGF promoter and expression of various bFGF isoforms in Kaposi sarcoma and endothelial cells. *AIDS*, 1998, 12:19.
13. Faris, M., N. Kokot, K. Latinis, S. Kasibhatla, D. Green, G. Koretzky and A.E. Nel. The JNK Cascade Plays a Role in Stress-induced Apoptosis in Jurkat Cells by Upregulating FasL Expression. *J. Immunol.*, 1998, 160:134.
14. Faris, M., B. Ensoli, J. Said, N. Kokot and A.E. Nel. Dominant active Ras affects the life-span, growth factor production and induces Kaposi's sarcoma characteristics in endothelial cells. *Cancer Res.*, 1997, submitted.
15. Ng, D., N. Kokot, M. Faris, A. Saxon and A. Nel. Macrophage Activation by Polycyclic Aromatic Hydrocarbons: Evidence for the involvement of stress-activation protein kinases, AP-1 and anti-oxidant response elements. *J. Immunol.*, 1998, 161: 942.
16. Faris, M., K. M. Latinis, S. Kempiaik, G. A. Koretzky and Andre Nel. Stress-Induced Fas Ligand Expression in T cells is Mediated Through A MEKK1-Regulated Response Element in the Fas Ligand Promoter. *Mol. Cell. Biol.*, 1998,18: 5414.
17. Shau, H., A.C. Huang, M. Faris, R. Nazarian., J. de Vellis and W. Chen. Thioredoxin peroxidase (natural killer enhancing factor) regulation of activator protein-1 function in endothelial cells. *Biochem. Biophys. Res. Commun.* 1998, 249: 683.
18. Abreu-Martin, M., A. Palladino, M. Faris, N. Carramanzana, A. Nel, and S.R. Targan. Fas Activates the JNK pathway in Human Colonic Epithelial Cells: Lack of a Direct Role on Apoptosis. *Am. J. Physiol.*, 1999, 276: 599.

ABSTRACTS:

1. M. Faris and B.S. Zwilling. Characterization of MHC Class II Expression by MacrophageHybrids. *J. Leuk. Biol.* 46: 314(86), 1989.
2. M. Faris and B.S. Zwilling. Regulation of the Induction of Persistent Ia Expression by Macrophages from Mice that are Resistant to Mycobacterium bovis Strain (BCG). *FASEB*, 4:1752, 1990.
3. M. Faris and B.S. Zwilling. Continuous Expression of MHC class II Glycoproteins by Macrophage-Hybrids: Regulation of the Induction of the Bcg Gene. *Proc. Biomed. Res. Society*, 1990.

4. B.S. Zwilling and M. Faris. Characterization of the Induction of Persistent I-A Expression by Macrophages from Bcg^r Mice. *J. Leuk. Biol.*, 48:52, 1990.
5. M. Faris and B.S. Zwilling. The Induction of Persistent Expression of MHC Class II (I-A) Glycoproteins is Mediated by Protein Kinase C (PK-C). *FASEB*, 5:5614, 1991.
6. M. Faris. The Induction of Persistent MHC class II Expression by rIFN- γ is Dependent on a Protein Kinase C Mediated Pathway. *The Graduate Research Forum (OSU)*, 1991.
7. M. Faris, F. Gaskin, R. S. Geha and S. M. Fu. Phosphorylation of a 28kD Protein by a CD40 Mediated Tyrosine Kinase Pathway. *J. Immunol.*, 150:556, 1993.
8. M. Faris, F. Gaskin, R. S. Geha and S. M. Fu. Tyrosine Phosphorylation Defines a Unique Transduction Pathway in Human B Cells Mediated via CD40. *Clinical Research*, 41:277A, 1993.
9. M. Faris and S.M. Fu. CD40 Signal Transduction: Association of CD40 with Lyn, PI3K, GAP and PLC γ . *Clinical Research*, 42:206A, 1994.
10. M. Faris, S. Wang and A. Nel. The Oncostatin M Induced Proliferative Response in Kaposi's Sarcoma Cells Involves Adaptor Proteins, Raf-1 and MEK-1. *Molecular Pathogenesis and Immunology of HIV-1*, 1994.
11. M. Faris, S. Wang, A. Nguyen and A. Nel. The Oncostatin M Response in Kaposi's Sarcoma Cells Involves JAKs, Adaptor Proteins, Raf-1 and MEK-1. *FASEB*, 9:202A, 1995.
12. A. Nel, M. Faris, F. Xu and N. Kokot. IL-4 and IL-6 Utilize Distinct JAK/Stat Pathways to Drive B-cell Differentiation as Determined at the Level of Ig Genes. *Cell Growth Symposium*, 1996.
13. M. Faris, N. Kokot, L. Lee and A.E. Nel. Regulation of IL-2 Transcription by the JNK Pathway in Jurkat Cells. *J. All. Clin. Immunol.*, 99: LB53, 1997.
14. M. Faris, N. Kokot, K. Latinis, G. A. Koretzky and A.E. Nel. Role of the JNK cascade in stress-induced apoptosis of Jurkat T cells. *FASEB J.*, 12:930A, 1998.
15. A.E. Nel, A. Saxon, D. Ng and M. Faris. Macrophage activation by polycyclic aromatic hydrocarbons: evidence for the involvement of stress-activated protein kinases, AP-1 and anti-oxidant response elements. *FASEB J.*, 12:1062A, 1998.
16. M. Faris, B. Goka and S. Stuart. Gene expression in breast cancer. *Clin. Chem.* 45: 10, 1999.
17. A. Raitano, I. Vivanco, R. Hubert, E. Chen, M. Faris, D. Saffran, D. Afar and A. Jakobovits. Auto-catalytic cleavage of the androgen regulated TMPRSS2 protease results in its secretion by prostate and colon cancer epithelia. *Proc. Amer. Assoc. Cancer Res.* 42: 657, 2001

18. M. Faris, P. Velasquez, R. Hubert, D. Saffran, A. Raitano and A. Jakobovits. Validation of STEAP-1 as a therapeutic target.
19. M. Faris, P. Velasquez, P. Nolan, R. Hubert, A. Raitano and A. Jakobovits. Validation of STEAP-1 as a Cell Surface Cancer Therapeutic Target. Proc. Amer. Assoc. Cancer Res. 43: 947, 2002.

PAPERS PRESENTED AT NATIONAL MEETINGS:

26th Annual Meeting, Society of Leukocyte Biology, October 15-18, 1989, Marco Island, FL, by M. Faris and B.S. Zwilling.

American Society for Biochemistry and Molecular Biology, The American Association of Immunologists Joint Meeting (FASEB), June 4-7, 1990, New Orleans, LA, by M. Faris and B.S. Zwilling.

27th Annual Meeting, Society of Leukocyte Biology, Twelfth International RES Congress, October 14-18, 1990, Heraklion, Crete, Greece by B.S. Zwilling and M. Faris.

Immunology of Mycobacterial Infections, National Jewish Center of Immunology and Respiratory Medicine, October 1990, Denver, Colorado, by B.S. Zwilling and M. Faris.

Federation of American Societies for Experimental Biology, April 21-25, 1991, Atlanta, GA, by M. Faris and B.S. Zwilling.

AAP/ASCI/AFCR Clinical Research Meeting, April 30-May 3, 1993, Washington DC by M. Faris, F. Gaskin, R.S. Geha and S.M. Fu.

American Association of Immunologists, The Clinical Immunology Society Joint Meeting (FASEB), May 21-25, 1993, Denver, Colorado, by M. Faris, F. Gaskin, R.S. Geha and S.M. Fu.

AAP/ASCI/AFCR Clinical Research Meeting, April 29-May 2, 1994, Baltimore, MD by M. Faris and S.M. Fu.

Federation of American Societies for Experimental Biology, April 9-13, 1995, Atlanta, GA by M. Faris, S. Wang, A. Nguyen and A. Nel.

UK-RSA Symposium on Cell Growth Control, January 28-February 1, 1996, Cape Town, RSA by A. Nel, M. Faris, F. Xu and N. Kokot.

AAAI/AAI/CIS Joint Meeting, February 21-26, 1997, San Francisco, by M. Faris, N. Kokot, L. Lee and A.E. Nel.

Federation of American Societies for Experimental Biology, April 18-22, 1998, San Francisco, by M. Faris, N. Kokot, K. Latinis, G. A. Koretzky and A.E. Nel

PAPERS PRESENTED AT LOCAL MEETINGS:

16th Annual ICSABER Graduate Research Forum, May 8, 1990, OSU, Columbus, OH, by M. Faris and B.S. Zwillling.

5th Annual Graduate Research Forum, April 20, 1991, Fawcett Center, Columbus, OH., by M. Faris.

12th Annual Research Day, Department of Medicine, April 26, 1993, OMNI Hotel, Charlottesville, VA, by M. Faris.

13th Annual Research Day in Internal Medicine, April 25, 1994, OMNI Hotel, Charlottesville, VA, by M. Faris.

Annual UCLA AIDS Institute Symposium: Molecular Pathogenesis and Immunobiology of HIV-1, November 11, 1994, Loews Santa Monica, CA by M. Faris.

SEMINARS:

Department of Microbiology, The Ohio State University, May 30, 1991. Induction of persistent MHC class II expression by macrophages.

Department of Rheumatology, University of Virginia, February 9, 1994. Update in CD40 mediated signaling: Involvement of PTK, PTP and PI3K.

Jonsson Cancer Center, UCLA, February 22, 1996. Involvement of the Stat pathway in B cell differentiation.

Department of Rheumatology, UCLA School of Medicine, October 30, 1996. Role of the JNK cascade in the regulation of IL-2 production in T lymphocytes.

Jonsson Cancer Center, UCLA, December 12, 1996. Regulation of IL-2 expression in Jurkat cells by MEKK1.

Jonsson Cancer Center, UCLA, October 28, 1997. Role of the JNK cascade in the apoptosis of T cells.

EuroCancer 1998, Paris, June 4, 1998. Integrated Approach to the Discovery of Cancer Therapeutics.

American Association of Clinical Chemists-Baychem 99, San Francisco, September 24, 1999. Expression Analysis of Cancer Genes.

PATENT APPLICATIONS:

1. Detection of Altered Expression of Genes Regulating Cell Proliferation. 11-1998.
2. Detection of EGF Regulated Genes in Breast Carcinomas. 9-1999.
3. Differential Gene Expression in Prostate Cancer. 2-2000
4. Prostate Cancer Markers. 2-2000.
5. GPCR Up-regulated in Prostate Cancer. 10-2000
6. 36P6D5: Secreted Tumor Antigen. 10-2000
7. 103P2D6: Tissue Specific Protein Highly Expressed in Various Cancers. 2-2000
8. Diagnosis and Therapy Using SGP28-Related Molecules. 10-2000
9. Novel Serpentine Transmembrane Antigens Expressed in Human Cancer. 12-1999
10. 83P5G4: a Tissue Specific Protein Highly Expressed in Prostate Cancer. 2-2000
11. 34P3D7: a Tissue. Specific Protein Highly Expressed in Prostate Cancer. 2-2000

HONORS AND AWARDS:

Honors Tuition Scholarship 1985-1986
ICSABER Graduate Forum Award, 1990.
AFCR Trainee Investigation Award, 1993.

GRANTS AND FUNDING

1. NIH-Tumor Immunology Training Grant, 1994. Title: Regulation of Signaling Pathways in Kaposi's Sarcoma. \$27,000.
2. NIH-Tumor Immunology Training Grant, 1995. Title: Regulation of Signaling Pathways in Kaposi's Sarcoma. \$29,000.
3. NIH Program Project Grant R and D 19, 1998. Title: Role of the JNK Pathway in SLE. \$60,000

PROFESSIONAL AFFILIATION:

Society for Leukocyte Biology.
American Federation for Clinical Research.
American Association for the Advancement of Science
American Association of Immunologists
Jonsson Cancer Center

CURRICULUM VITAE:

KAREN JANE MEYRICK MORRISON

(A)

TRAINING/QUALIFICATIONS:

- 1998 PhD., Department of Pathology, University of
 Southampton, U.K.
 Title of thesis: An investigation of inflammatory cells
 in asthma as studied by immunocytochemical
 techniques on bronchial biopsies.
- 1985 Fellow, Institute of Biomedical Sciences, U.K.
- 1979 Associate, Institute of Biomedical Sciences, U.K.
- 1978 BSc. (Hons.) 2.2 Zoology,
 University of Southampton, U.K.

EMPLOYMENT

PRESENT EMPLOYMENT:

April 2001 – present Research Scientist, Agensys, Inc., 1545 Seventeenth Street,
 Santa Monica, CA 90404.

PREVIOUS EMPLOYMENT:

July 1994 - B.M.S. 3 (Biomedical Scientist 3), Cardiothoracic
January 2001 Surgery, Imperial College School of Medicine at Harefield Hospital, Harefield,
 U.K.

September 1993 - Research Associate, Smooth Muscle Group,
July 1994 U.M.D.S., St. Thomas's Hospital, London, U.K.

June 1992 - Research Associate, Department of Medicine,

August 1993	University of Southampton at Southampton General Hospital, Southampton, U.K.
October 1988 - May 1992	Research Assistant, Department of Medicine, University of Southampton at Southampton General Hospital, Southampton, U.K.
September 1987 - October 1988	B.M.S. 2, Department of Histopathology, Royal Victoria Hospital, Boscombe, Bournemouth, U.K.
January 1986 - September 1987	B.M.S. 2, Department of Pathology, University of Southampton at Southampton General Hospital, Southampton, U.K.
November 1979 - January 1986	B.M.S. 1, Department of Pathology, University of Southampton at Southampton General Hospital, Southampton, U.K.
July 1978 - November 1979	Junior 'B' B.M.S., Department of Pathology, University of Southampton at Southampton General Hospital, Southampton, U.K.

EXPERIENCE:

- General histology. All general histological techniques including the sample preparation, processing, cutting and staining of sections from a variety of frozen, paraffin and resin embedded tissue.
- Immunohistochemistry. Extensive knowledge of numerous immunohistochemical techniques in a variety of tissue and cell preparations. These include the development and adaptation of various methods in frozen, paraffin and resin embedded preparations.
- Quantitative techniques. Methods for the evaluation of cells and tissue sections using both manual and computer-aided systems.
- In situ hybridisation. The use of non-radiolabeled techniques for the demonstration of mRNA in tissue sections.
- Responsibility. Instrumental in the establishment and day to day running of immunohistochemistry and general histology units previously for the Department of Medicine, Southampton General

Hospital; Smooth Muscle Group, St Thomas's Hospital and Department of Cardiothoracic Surgery, Imperial College at Harefield Hospital and in the current post

- Training and supervision. The training and supervision of undergraduate and PhD students, biomedical scientists, academic research staff and clinicians undertaking projects requiring histological techniques. Teaching immunohistochemistry to visitors from other research institutions both in the U.K. and abroad.
- Computer literacy. Literate in a broad spectrum of software including Office, image analysis and statistical packages.

KAREN JANE MEYRICK MORRISON

PUBLICATIONS:

Judd MA and **Britten (now Morrison) KJM**. (1982) Tissue preparation for the demonstration of surface antigens by immunoperoxidase techniques. *Histochemical Journal* 14 : 747 - 753.

Stratford N, **Britten KJM** and Gallagher PJ. (1985) Inflammatory infiltrates in human coronary atherosclerosis. *Atherosclerosis* 59 : 271 - 276.

Jones DB, **Britten KJM**, de Sousa M and Wright DH. (1985) The distribution of ferritin and ferric iron in the spleens of lymphoma patients and controls. In: *Proteins of the Biological Fluids, Volume 32*. Eds: H Reefers. Pergamon Press: Oxford.

Jones DB, **Britten KJM** and Wright DH. (1986) The staining of a panel of routine diagnostic tissue biopsies with workshop 'L' series antibodies. In: *Leucocyte typing, Volume 2, Chapter 24*. Eds; Reinherz and Nadler. Springer-Verlag: Berlin.

Britten KJM, Jones DB, de Sousa M and Wright DH. (1986) The distribution of iron and iron binding proteins in spleen with reference to Hodgkin's disease. *British Journal of Cancer* 54 : 277 - 286.

Mephram BL and **Britten KJM**. (1990) Immunocytochemical techniques in lymphoreticular pathology. In: *Lymphoproliferative Diseases, Chapter 12*. Eds: Jones and Wright. Kluwer Academic Publishers: London.

Djukanovic R, Wilson JW, **Britten KJM**, Wilson SJ, Walls AF, Roche WR, Howarth PH and Holgate ST. (1990) Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *American Review of Respiratory Disease* 142 : 863 - 871.

Holgate ST, Djukanovic R, Wilson JW, Roche WR, **Britten KJM** and Howarth PH. (1991) Allergic inflammation and its pharmacological modulation in asthma. *International Archives of Allergy and Applied Immunology* 94 : 210 - 217.

Howarth PH, Wilson JW, Djukanovic R, Wilson SJ, **Britten KJM**, Walls AF, Roche WR and Holgate ST. (1991) Airway inflammation and atopic asthma: a comparative bronchoscopic investigation. *International Archives of Allergy and Applied Immunology* 94 : 266 - 269.

Djukanovic R, Wilson JW, **Britten KJM**, Wilson SJ, Walls AF, Roche WR, Howarth PH and Holgate ST. (1992) Effect of inhaled corticosteroid on airway inflammation and symptoms in asthma. American Review of Respiratory Disease 145 : 669 - 674.

Djukanovic R, Lai CKW, Wilson JW, **Britten KJM**, Wilson SJ, Walls AF, Roche WR, Howarth PH and Holgate ST. (1992) Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic non-asthmatics and healthy controls. European Respiratory Journal 5 : 538 - 544.

Montefort S, Roche WR, Howarth PH, Djukanovic R, Gratziau C, Carroll MP, Smith L, **Britten KJM**, Haskard DO, Lee TH and Holgate ST (1992). Interleukin-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) expression in the bronchial mucosa of normal and asthmatic subjects. European Respiratory Journal 5 : 815 - 823.

Bradding P, Feather IH, Howarth PH, Mueller R, Roberts JA, **Britten KJM**, Bews JPA, Hunt TC, Okayama Y, Heusser CH, Bullock GR, Church MK and Holgate ST. (1992) Interleukin 4 is localised to and released by human mast cells. Journal of Experimental Medicine 176 : 1381 - 1386.

Britten KJM, Howarth PH and Roche WR. (1993) Immunohistochemistry on resin sections. A comparison of resin embedding techniques for small mucosal biopsies. Biotechnic and Histochemistry 68 : 271 - 280.

Bradding P, Roberts JA, **Britten KJM**, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH and Holgate ST. (1994) Interleukins 4, 5 and 6 and tumour necrosis factor α in normal and asthmatic airways: evidence for the human mast cell as an important source of these cytokines. American Journal of Respiratory Cell and Molecular Biology 10 : 471 - 480.

Amrani M, Latif N, **Morrison K**, Jayakumar N, Goodwin A, Gray C, Dunn M and Yacoub M. (1998) Relative induction of heat shock protein (HSP70) in coronary endothelial cells versus cardiomyocytes. Implications for myocardial protection. Journal of Thoracic and Cardiovascular Surgery 115 : 200 - 209.

Chester AH, Borland JAA, **Morrison KJM**, Amrani M, Thom S McG and Yacoub MH. (1998) Reactivity of small intra-myocardial arteries from atherosclerotic and non-atherosclerotic human hearts. Journal of Vascular Research 35 : 170 - 178.

Allen S, Dashwood M, **Morris** and Yacoub MH. (1998) Differential leukocyte constrictor responses in human atherosclerotic coronary arteries. *Circulation* 97 : 2406 - 2413.

Borland JA, Chester AH, **Morrison KJM** and Yacoub MH. (1998) Alternative pathways of angiotensin II production in the human saphenous vein. *British Journal of Pharmacology* 125 : 423 - 428.

Chester AH, **Morrison KJM** and Yacoub MH. (1998) Expression of vascular adhesion molecules in saphenous vein coronary bypass grafts. *Annals of Thoracic Surgery* 65 : 1685 - 1689.

Petrou M, Clarke S, **Morrison K**, Bowles C, Dunn M and Yacoub M. (1999) Clenbuterol increases specific power and twitch speed of skeletal muscle for cardiac assist. *Circulation* 99 : 713 - 720.

Ensminger SM, Witze O, Spriewald BM, **Morrison K**, Morris PJ, Rose ML and Wood KJ (2000). CD8+ T cells contribute to the development of transplant arteriosclerosis despite CD154 blockade. *Transplantation* 69 : 2609 – 2612.

Ensminger SM, Spriewald BM, Witze O, **Morrison K**, Morris PJ, Rose ML and Wood KJ (2000). Intragraft IL-4 expression following CD154 blockade may trigger delayed development of transplant arteriosclerosis in the absence of CD8+ T cells. *Transplantation* 70 : 955 - 963.

Raisky O, **Morrison KJM**, Obadia JF, McGregor J, Yacoub MH and Rose ML (2001). Acute rejection and cardiac graft vasculopathy in the absence of donor derived ICAM-1 or P-selectin. *Journal of Heart and Lung Transplantation* 20 : 340 – 349.

Abusnara HJ, Smolenski RT, **Morrison K**, Yap J, Sheppard MN, O'Brien T, Suzuki K, Jayakumar J and Yacoub MH (2001). Efficacy of adenoviral gene transfer with manganese superoxide dismutase and endothelial nitric oxide synthase in reducing ischemia and reperfusion injury. *European Journal of Cardiothoracic Surgery* 20 : 153 – 158.

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representation of
The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

(B) + (G)

Expression of Epithelial Mucins MUC1, MUC2, and MUC3 in Ductal Carcinoma In Situ of the Breast

Leslie K. Diaz, MD,* Elizabeth L. Wiley, MD,* and Monica Morrow, MD†

*Departments of *Pathology and †Surgery, Lynn Sage Breast Program,
Northwestern University Medical School, Chicago, Illinois*

■ **Abstract:** Epithelial mucins are glycoproteins secreted by epithelial cells and their carcinomas. At least nine mucin genes have been identified, and their products (MUC1–MUC9) are expressed in various epithelia. MUC1 is a mucin expressed in breast epithelial cells, whereas MUC2 and MUC3 are primarily intestinal mucins. Although MUC1 and MUC2 expression has been documented in invasive ductal carcinoma of the breast, mucin expression in pure ductal carcinoma in situ (DCIS) has not been investigated. Sixty-one of 105 cases of DCIS without coexisting infiltrating carcinoma diagnosed during a 30-month period were selected as having sufficient tissue for study. Paraffin-embedded tissue sections were stained using immunohistochemical techniques with mouse monoclonal anti-MUC1, anti-MUC2, and rabbit-specific polyclonal anti-MUC3 antibodies. Immunoreactive epitopes of MUC1, MUC2, and MUC3 were expressed in DCIS in 61, 19, and 16 of 61 cases, respectively. MUC2 and MUC3 staining intensity in DCIS was markedly less than that observed for MUC1. Luminal and/or cytoplasmic patterns of staining were observed for MUC1. MUC2 and MUC3 showed only cytoplasmic staining. Cytoplasmic-only staining of MUC1 was associated with a higher grade of DCIS. Any MUC2 staining was also associated with a higher grade of DCIS. Coexpression of MUC2 and MUC3 was present in only 6 of 61 cases, and MUC3 staining was unrelated to the grade of DCIS. Cytoplasmic expression of MUC1 and MUC2 appears to be associated with a higher grade of DCIS. MUC3 ex-

pression appears to be independent of grade and expression of MUC1 and MUC2. The relationship of mucin expression and grade warrants further study. ■

Key Words: breast, ductal carcinoma in situ, epithelial membrane antigen, immunohistochemistry, mucin

E epithelial mucins are transmembrane glycoproteins that are produced by both normal epithelial cells and malignant epithelial tumors of pulmonary, gastrointestinal, gynecologic, and mammary origin. Mucins are complex molecules ranging in size from 400 kDa to more than 1,000 kDa. They are a heterogeneous group of molecules whose variations in molecular structure are thought to carry tissue-specific functions (1–4). Nine such mucins, MUC1–MUC9, have had their genes identified and their respective products either completely or partially characterized (5,6). All mucins are characterized by a tandemly paired and repetitive central peptide that is rich in serine and threonine. The peptides have little to no homology among mucin types and are thus ideal epitopes for raising type-specific antibodies to mucins (2,4,7). MUC1, also known as epithelial membrane antigen, is the most extensively studied of the mucins (8). Although it was originally described as a tissue-specific glycoprotein of breast epithelium, MUC1 is present in most polarized epithelial cells (7–12). The expression of other secretory mucin may be localized to specific tissue types. MUC2 and MUC3 are thought to be prima-

Address correspondence and reprint requests to: Leslie K. Diaz, MD, Department of Pathology, Feinberg Pavilion 7-325, 251 E. Huron St., Chicago, IL 60611.

rily expressed in gastrointestinal tissues. MUC4, MUC5, and MUC8 are found in bronchial tissues. MUC7 is found in salivary gland and MUC6 in gastric epithelium (13-17). MUC2, MUC3, MUC5, and MUC6 have highly conserved cysteine domains which are thought to form disulfide bonds between mucin monomers, accounting for the high viscosity of these mucins (18). The overall tissue distribution of mucins in normal and malignant epithelial cells has not been completely elucidated (13-16).

Likewise the patterns of mucin expression in benign and malignant breast tissue have not been completely established. MUC1 is the only mucin that characteristically is expressed by normal breast epithelium (5,8,19). Several studies have focused on MUC1, MUC2, and MUC5 expression in invasive carcinomas of the breast (13,16-18) and have noted expression of these mucins in coexisting normal and atypical duct epithelium and DCIS (17,18). MUC1 has been shown to be overexpressed in invasive ductal carcinoma and is thought to facilitate metastatic behavior (8). MUC2 and MUC5 are found in colloid carcinomas of the breast and are variably present in adjacent benign epithelium (11,16,18). MUC2 has been shown to be absent in most nonmucinous ductal carcinomas of the breast. Overexpression of MUC2 by infiltrating ductal carcinomas has been associated with more aggressive behavior than those without (17). The preferred site of expression for MUC3 is the absorptive cell of the small intestine (6). The presence of MUC3 in normal breast epithelium has not been reported.

Mucin expression in DCIS without concurrent invasive disease (so-called pure DCIS) has not been previously studied. Characterizing the expression of different epithelial mucins in DCIS would help to determine a baseline rate of mucin expression and delineate the differences between normal ductal epithelial cells and those that form this important precursor lesion of invasive breast carcinoma. The primary focus of this study is to compare the expression of MUC1 with MUC2 and MUC3 in cases of pure DCIS, where no invasive carcinoma exists, and the adjacent nonmalignant breast epithelium.

MATERIALS AND METHODS

Clinical histories and slides from consecutive patients with a diagnosis of DCIS were reviewed. Cases with insufficient in situ tumor for study and cases with coexisting infiltrating carcinoma were excluded; 105 cases were found to meet the above requirements. From each

Table 1. MUC1, MUC2, and MUC3 Staining Patterns for 61 Cases of Pure DCIS

Staining pattern	MUC1	MUC2	MUC3
None	0	42	45
Cytoplasmic only	26	19	16
Combined	26	0	0
Luminal only	9	0	0

case, two blocks containing DCIS were sectioned at 3 μ m intervals and mounted on positively charged glass slides for a total of 12 unstained slides. The first and last sections were stained with hematoxylin and eosin to confirm the presence of lesional tissue. Forty-four cases did not have lesional DCIS maintained in the cut sections and these were excluded from the study. The intervening sections from the remaining 61 cases were stained for MUC1, MUC2, and MUC3.

The immunohistochemical method is as follows: Unstained sections were deparaffinized and hydrated using graded xylene and alcohol solutions. Sections were stained using an automated stainer with an avidin-biotin peroxidase system. Sections were incubated with mouse anti-MUC1 (clone B24.1, Biomedica Corp., Foster City, CA), mouse anti-MUC2 (clone B306.1, Biomedica), and rabbit polyclonal anti-MUC3 (Biomedica). Staining dilutions were 0.01 μ g/ml for MUC1, 0.01 μ g/ml for MUC2, and 0.02 μ g/ml for MUC3. Negative controls used nonimmune serum on four cases. Tissue sections of stomach and breast were used as controls as suggested by the manufacturer.

Stained sections were reviewed by two authors for type, pattern, and amount of mucin positivity. Sections were scored for percentage of carcinoma in situ cell staining and the location of staining within positive cells. Cells were considered staining positive if the brown pigment of diaminobenzidine could be readily detected at scanning magnification. The locations of staining consisted of luminal (staining of the duct luminal surface), membranous (staining of the entire cyto-

Table 2. MUC1 Staining Compared to Grade of DCIS

	Grade of DCIS			Total
	1	2	3	
Luminal only	3	6	0	9
Combined luminal cytoplasmic	8	11	7	26
Cytoplasmic only	1	6	19	26
Total for grade	12	23	26	61

plasmic border), and cytoplasmic (granular staining of cell cytoplasm). The percentage of DCIS or epithelial cells staining was scored as 0, focal, and positive as follows: 0 (0% cells staining), focal (any cells up to 5% of tumor cells), and positive (greater than 5% of tumor cells). Tumor cells were compared to the epithelial staining present in adjacent benign breast tissue. Results were compared using Propher (BBN Systems and Technologies), a statistics program sponsored by the National Institutes of Health, using chi-squared 2×2 -test.

RESULTS

Of the 61 cases of DCIS, 12 were grade 1 and were predominately cribriform or solid patterns. Twenty-four cases were grade 2 and were cribriform or solid with necrosis. The remaining 25 DCIS cases were grade 3 and were predominately solid with comedo-type necrosis.

Table 1 summarizes the number of DCIS cases staining for each mucin type and the pattern of staining. Sixty of 61 cases (98%) demonstrated positive staining for MUC1 and 1 case was focally positive. Twenty-six cases (40%) had cytoplasmic-only staining, 9 cases (15%) showed only luminal staining, and 26 cases (40%) had combined cytoplasmic and membranous staining. Benign breast epithelium in 42 of 61 cases showed a luminal-only pattern of staining for MUC1. MUC1 staining was less intense in the benign epithelium of the same case when compared to staining of DCIS for the same case. Table 2 shows the distribution of MUC1 staining compared to the grade of DCIS. Nineteen of 26 grade 3 DCIS cases had only cytoplasmic staining for MUC1 compared to 7 of 35 grade 1 and 2 cases ($p = 0.002$).

Nineteen cases of DCIS (32%) stained with antibody to MUC2 and all of these cases demonstrated a cytoplasmic pattern of staining. Of these, 8 had only focal staining and 11 were considered positive with more than 5% of tumor cells staining. Benign breast epithelium in two cases had focal staining for MUC2; the remaining 59 showed no staining with anti-MUC2 antibody. Both cases with staining of benign epithelium had coexisting

Table 4. Staining for MUC3 Compared to Grade of DCIS

	Grade of DCIS			Total
	1	2	3	
No MUC3 staining	10	16	19	45
Focal MUC3 staining	1	3	5	9
Positive MUC3 staining	1	5	1	7
Total for grades	12	24	25	61

MUC2-positive in situ lesions. Compared to staining for MUC1, MUC2 staining of tumor cells was observed to be less intense than that seen for MUC1 staining of DCIS. Table 3 shows the distribution of staining for MUC2 compared to the grade of DCIS. Twelve of the 19 cases with any staining for MUC2 were grade 3 in situ carcinomas ($p < 0.006$).

Sixteen cases of DCIS stained with polyclonal antibody to MUC3; of these 9 cases had only focal staining of tumor cells and 7 cases had greater than 5% of tumor cells staining. All 16 cases demonstrated a cytoplasmic pattern of staining. Adjacent benign breast epithelium demonstrated faint (background quality) staining in 53 cases in a cytoplasmic in pattern; the optical intensity was insufficient for positive staining. The distribution of staining for MUC3 compared to DCIS grade is shown in Table 4. A correlation with MUC3 positivity and high grade was not observed.

Table 5 lists the distribution of staining for MUC2 compared to MUC3. Most cases that had staining for MUC2 were negative for MUC3. Twenty-nine cases had staining for MUC2 and/or MUC3, but only six cases showed staining for both mucins, with one or both mucins showing only focal staining.

DISCUSSION

The results of this study establish a rate of expression in pure DCIS for three mucins: MUC1, MUC2, and MUC3. MUC1 staining was observed in every case of DCIS (Fig. 1) and was expressed strongly in all but a single case. Three staining patterns were present for MUC1:

Table 3. Staining for MUC2 Compared to Grade of DCIS

	Grade of DCIS			Total
	1	2	3	
No staining for MUC2	9	20	13	42
Focal positive MUC2	0	2	6	8
Positive MUC2	3	2	6	11
Total for grade	12	24	25	61

Table 5. Staining of MUC2 Compared to MUC3 for 61 Cases of DCIS

	Staining for MUC2			Total
	Negative	Focal	Positive	
MUC3 negative	32	5	8	45
Focal MUC3	4	2	3	9
Positive MUC3	6	1	0	7
Total	42	8	11	61



Figure 1. DCIS stained with monoclonal antibody to mucin type 1 (MUC1); staining is present in both the cytoplasm of the in situ carcinoma lining the ducts and variably at the luminal surface of the ducts. (Diaminobezidine with hematoxylin counterstain; original magnification $\times 200$.)



Figure 2. DCIS stained with monoclonal antibody to mucin type 2 (MUC2); variable granular cytoplasmic staining is present in many of the cells. (Diaminobezidine with hematoxylin counterstain; original magnification $\times 200$.)

luminal only, cytoplasmic only, and combined luminal cytoplasmic. MUC1 is the only mucin currently described as a cytoplasmic-membrane bound molecule and this property explains the observed luminal position of staining in the cases studied (5,8). MUC1 expression was present not only in DCIS but also in adjacent benign breast epithelium. Stronger MUC1 staining was generally seen in DCIS compared to the adjacent benign epithelium. This pattern of MUC1 expression is similar to that described for infiltrating carcinomas (8,9). Also the loss of luminal expression of MUC1 with only cytoplasmic MUC1 was associated with higher-grade DCIS. This benign and neoplastic staining pattern for MUC1 parallels that recently described in the pancreas by Monges et al. (19) where primarily apical (luminal) staining is expressed in benign pancreatic acini compared to cytoplasmic MUC1 expression present in ductal adenocarcinomas.

MUC2 expression (Fig. 2) was observed in only 28% (19 of 61) of cases of DCIS. MUC2 is characterized as a gel-forming protein and this correlated with a purely cytoplasmic staining location of this mucin that was ob-

served in this study. Expression of MUC2 in adjacent benign breast tissue was weak to absent, suggesting that MUC2 is relatively DCIS specific when positive staining is detected. Like cytoplasmic expression of MUC1, MUC2 expression in DCIS was strongly associated with higher grade. Although MUC2 has been shown to be strongly expressed by invasive colloid carcinoma, a low-grade tumor in breast (16,18), MUC2 positivity has been correlated with more aggressive tumor behavior and poorer prognosis in infiltrating ductal carcinomas when compared to MUC2-negative tumors. The association of MUC2 expression with higher-grade DCIS would parallel this.

This is the first study that the authors are aware of which describes MUC3 expression in breast epithelium. We found that the pattern of staining for MUC3 in DCIS and benign breast epithelium was similar to that of MUC2 (Fig. 3). However, only 6 of 61 cases showed coexpression of MUC2 and MUC3. MUC3 expression did not show a correlation with the grade of DCIS as was found for MUC2 and MUC1.

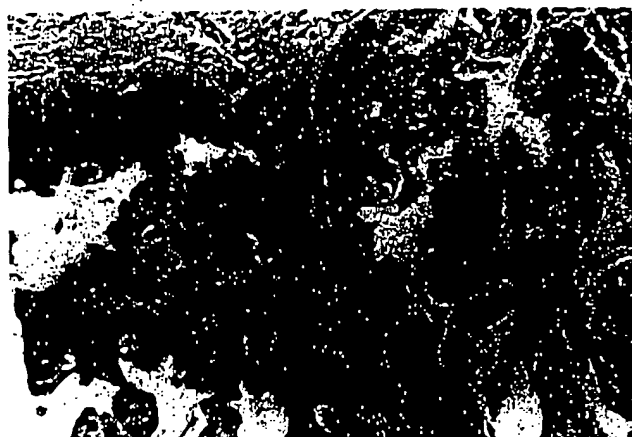


Figure 3. Polyclonal antibody raised against mucin type 3 is used to stain cells of DCIS. Scattered cells show heavy cytoplasmic staining, sharply defining them against adjacent negatively staining cells. (Diaminobezidine with hematoxylin counterstain; original magnification $\times 330$.)

This study, using cases of pure DCIS, showed that DCIS has patterns of MUC1 and MUC2 expression similar to those described for invasive carcinoma. Of interest is the observation that MUC2 is expressed in 19 of 61 (31%) DCIS cases studied and in benign tissue in only 2 of 61 cases. This suggests that expression of this mucin is a marker of neoplasia in breast tissue. Cytoplasmic expression of MUC1 also appears to mark neoplastic change in DCIS, in that the mechanism of membrane attachment of MUC1 appears to be absent or defective in a portion of in situ carcinomas. Immunoreactive MUC3 expression of neoplastic breast epithelium using polyclonal antibody does not appear to correlate with MUC2 expression, MUC1 cytoplasmic expression, or grade of DCIS. Its expression was in a minority of DCIS cases in this study, and whether it is expressed in invasive carcinoma needs to be further investigated, preferably using monoclonal antibodies as they become available for study.

Potential clinical applications utilizing specific mucin epitopes of neoplastic breast diseases range from diagnostic to therapeutic. CA 15-3 (MUC1) is currently used to monitor response to breast cancer therapy. Radiolabeled anticancer antibodies against breast-specific mucins may be utilized for high-resolution imaging as well as tumor-directed therapy, similar to antibody against HER-2/*neu* (20,21). Mucin protein and carbohydrate epitopes are strong candidates for a tumor vaccine that may someday target breast cancer cells specifically.

MUC1 overexpression and MUC2 expression are found to occur in higher-grade in situ lesions. Kanthan

et al. (22) recently noted that aberrant mucin expression may be an early step in oncogenesis. These findings and those of this study serve to validate the use of grade as a marker of biologic behavior. The differential mucin expressions are worthy of further investigation to determine if they will prospectively allow the identification of duct carcinoma lesions at risk for invasion or recurrence.

Acknowledgment

Supported by a grant from the Lynn Sage Cancer Research Foundation of Northwestern Memorial Hospital, Chicago, Illinois.

REFERENCES

1. Gum JR. Mucins. Their structure and biology. Glycobiology Group Colloquium. Meeting held at the University of Manchester, July 1995.
2. Kim YS, Gum J, Brockhausen L. Mucin glycoproteins in neoplasia. *Glycoconjugate J* 1996;13:693-707.
3. Bartman AE, Buisine MP, Aubert JP, et al. The MUC6 secretory mucin gene is expressed in a wide variety of epithelial tissues. *J Pathol* 1998;186:398-405.
4. Devine PL, McGuckin MA, Birrell GW, et al. Monoclonal antibodies reacting with MUC2 mucin core protein. *Br J Cancer* 1993;67:1182-88.
5. Gendler SJ, Spicer AP. Epithelial mucin genes. *Annu Rev Physiol* 1995;57:607-34.
6. Seregini E, Borti C, Massaron S, et al. Structure, function, and gene expression of epithelial mucins. *Tumori* 1997;83:625-32.
7. DeBolos C, Guma M, Barranco C, et al. MUC6 expression in breast tissues and cultured cells: abnormal expression in tumors and regulation by steroid hormones. *Int J Cancer* 1998;77:193-99.
8. Segal-Eiras A, Croce MV. Breast cancer associated mucin: a review. *Allergol Immunopathol* 1997;25:176-81.
9. Arklie J, Taylor-Papadimitriou J, Bodmer W, et al. Differentiation antigens expressed by epithelial cells in lactating breast are also detectable in breast cancer. *Int J Cancer* 1981;28:23-29.
10. Shimizu M, Yamauchi K. Isolation and characterization of mucin-like glycoprotein in human milk fat globule membrane. *J Biochem* 1982;91:515-24.
11. Cordell J, Richardson TC, Pulford KA, et al. Production of monoclonal antibodies against human epithelial membrane antigen for use in diagnostic immunohistochemistry. *Br J Cancer* 1985;52:347-54.
12. Pinkus GS, Kurtin PJ. Epithelial membrane antigen: a diagnostic discriminant in surgical pathology. *Hum Pathol* 1985;16:929-40.
13. Hanski C, Hofmeier M, Schmitt-Graff A, et al. Overex-

pression or ectopic expression of MUC2 is the common property of mucinous carcinomas of the colon, pancreas, breast, and ovary. *J Pathol* 1997;182:385-91.

14. Dong Y, Walsh MD, Cummings MC, *et al.* Expression of MUC1 and MUC2 mucins in epithelial ovarian tumours. *J Pathol* 1997;183:311-17.

15. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. *Int J Biochem Cell Biol* 1998;30:797-801.

16. O'Connell JT, Shao Z, Drori E, *et al.* Altered mucin expression is a field change that accompanies mucinous (colloid) breast carcinoma histogenesis. *Hum Pathol* 1998;29:1517-22.

17. Walsh MD, McGuckin MA, Devine PL, Hohn BG, Wright RG. Expression of MUC2 epithelial mucin in breast carcinoma. *J Clin Pathol* 1993;46:922-25.

18. Yonezawa S, Nomoto M, Matsukita S, *et al.* Expression of MUC2 gene product in mucinous carcinoma of the breast: comparison with invasive ductal carcinoma. *Acta Histochem Cytochem* 1995;28:239-46.

19. Monges M, Mathoulin-Portier MP, Acres RB, *et al.* Differential MUC1 expression in normal and neoplastic human pancreatic tissue. *Am J Clin Pathol* 1999;112:635-40.

20. Kim YS. Mucin glycoproteins in colonic neoplasia. *Keio J Med* 1998;47:10-18.

21. Goldberg DM, Nabi HA. Breast imaging with radiolabeled antibodies. *Semin Nucl Med* 1999;29:41-48.

22. Kanthan R, Xiang J, Magliocco AM. P53, ErbB2, and TAG-72 expression in the spectrum of ductal carcinoma in-situ of the breast classified by the Van Nuys system. *Arch Pathol Lab Med* 2000;124:234-39.



Selection of Tumor Antigens as Targets for Immune Attack Using Immunohistochemistry: Protein Antigens^{1,2}

Shengle Zhang, Helen S. Zhang,
Carlos Cordon-Cardo, Govindaswami Ragupathi,
and Philip O. Livingston³

Departments of Medicine [S. Z., H. S. Z., G. R., P. O. L.] and
Pathology [C. C. C.], Memorial Sloan-Kettering Cancer Center, New
York, New York 10021

ABSTRACT

The relative expression of mucin antigens MUC1, MUC2, MUC3, MUC4, MUC5_{AC}, MUC5_B, and MUC7 and glycoprotein antigens KSA, carcinoembryonic antigen, prostate-specific membrane antigen (PSMA), HER-2/*neu*, and human chorionic gonadotropin- β on different cancers and normal tissues is difficult to determine from available reports. We have compared the distribution of these antigens by immunohistochemistry on a broad range of malignant and normal tissues. MUC1 expression was most intense in cancers of breast, lung, ovarian, and endometrial origin; MUC2 was most intense in cancers of colon and prostate origin; and MUC5_{AC} was most intense in cancers of breast and gastric origin. MUC4 was intensely expressed in 50% of cancers of colon and pancreas origin, and MUC3, MUC5_B, and MUC7 were expressed in a variety of epithelial cancers, but not so intensely. KSA was intensely and uniformly expressed on all epithelial cancers; carcinoembryonic antigen was expressed in most cancers of breast, lung, colon, pancreas, and gastric origin; and PSMA was expressed only in cancers of prostate origin. Human chorionic gonadotropin- β was expressed on the majority of sarcomas and cancers of breast, lung, and pancreas origin, although intense staining was not seen. Staining on normal tissues was restricted to one or many normal epithelial tissues ranging from MUC3, MUC4, and PSMA, which were expressed only on epithelia of pancreas, stomach, and prostate origin, respectively, to MUC1 and KSA, which were expressed on most normal epithelia. Expression was restricted to the secretory borders of these epithelia while stroma and other normal tissues were completely negative. These results plus the results of the two previous papers (S. Zhang *et al.*, *Int. J. Cancer*, 73: 42-49,

1997; S. Zhang *et al.*, *Int. J. Cancer*, 73: 50-56, 1997) in this series provide the basis for selection of multiple cell surface antigens as targets for antibody-mediated attack against these cancers.

INTRODUCTION

This is our third and final immunohistochemistry study comparing the expression of a series of cell surface antigens (selected as potential targets for immunotherapy) on a range of normal and malignant tissues. The previous two studies (1, 2) focused on carbohydrate epitopes expressed in glycolipids, mucins, and other glycoproteins. Here, we focus on the peptide epitopes of seven mucins and five glycoproteins, each of which is available for vaccine construction as a consequence of simple peptide synthesis (MUC1-MUC7) or expression in *Escherichia coli* or baculovirus (3-7). Each of these antigens is either known to be expressed at the cell surface as a consequence of a demonstrated transmembrane domain (MUC1, KSA, CEA,⁴ PSMA, and HER-2/*neu*; Refs. 8-12) or is thought to be shed by tumor cells and be either adherent to or abundant in the vicinity of tumor cells (MUC2, MUC3, MUC4, MUC5_{AC}, MUC5_B, and MUC7 and β hCG; Refs. 13-19). Although the expression of each of these antigens on human tumors and normal tissues has been described, previous studies were limited in terms of number and types of tissues studied, involved mAbs against only one to three antigens without direct comparison to expression of other antigens, and used different immunostaining procedures (indirect immunofluorescence, indirect immunoperoxidase, or ABC immunoperoxidase; Refs. 13 and 19-29). Consequently, the comparative distribution of these antigens on cancers and normal tissues is difficult to determine from available reports, although this is precisely the information required for selecting target antigens for immunotherapy. This is especially important with the recent development of conjugate vaccines capable of inducing antibodies in most patients against a variety of well-defined tumor antigens (30) and with the recent evidence that the induction of these antibodies correlates with a more favorable prognosis (30-32).

MATERIALS AND METHODS

Tissue Samples. Frozen specimens embedded in Tissue-Tek O.C.T. compound (Diagnostic Division, Elkhart, IN) were provided with pathological reports by the Tissue Procurement Service of Memorial Sloan-Kettering Cancer Center (New York, NY), with the exception of four frozen specimens of metastatic prostate cancer, which were kindly provided by Dr.

Received 6/12/98; revised 8/20/98; accepted 8/31/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This paper is the third in a series. See Refs. 1 and 2.

² This work was supported by NIH Grants RO1 CA 61422 and PO1 CA 33049 and the United States Army DAMD 17-97-1-7115.

³ To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-7425; Fax: (212) 794-4352.

⁴ The abbreviations used are: CEA, carcinoembryonic antigen; β hCG, human chorionic gonadotropin- β ; mAb, monoclonal antibody; ABC, avidin-biotin complex; GI, gastrointestinal; PSMA, prostate-specific membrane antigen.

2670 Selection of Tumor Antigens for Immune Attack: Proteins

Table 1 Mouse mAbs used for immunohistology

mAb	Ig class	Antigen	Antigen structure	Ref.
HMFG-2	IgG1	MUC1	VTSAPDTRPAPGSTAPPAHG repeating	41, 42
LDQ10	IgM	MUC2	PTTTPISTTTTVPITPTPIGTQT repeating	37
M3.2	IgG2a	MUC3	HSTPSFTSSITTTETTS repeating	20
MUC4.275	IgG1	MUC4	TSSASTGHATPLPVTQ repeating	43
CLH2	IgG1	MUC5 _{AC}	TTSTTSAP repeating (interrupted)	27
PANH2	IgG1	MUC5 _n	No peptide repeats	44, 45
PANH3	IgG1	MUC7	TTAAPTTPSATTPAPPSSSAPPE repeating	44, 45
NCL-CEA	IgG1	CEA	Glycoprotein (M 180,000)	Vector Laboratories
Cyt351	IgG	PSMA	Glycoprotein (M 100,000)	28, 46
GA733-2	IgG2a	KSA	Glycoprotein (M 40,000)	47
FB12	IgG1	βhCG	145-amino acid glycoprotein	48
NCL-CBE1	IgG2a	HERv2/neu	Protein (M 185,000)	8

Table 2 Proportion of cancer specimens with ≥50% positive cancer cells (≥2+ staining intensity) by immunohistology*

Cancer	Antigen (mAb)											
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (M3.2)	MUC4 (M4.275)	MUC5 _{AC} (CLH2)	MUC5 _n (PANH2)	MUC7 (PANH3)	KSA (GA733-2)	PSMA (Cyt351)	CEA (NCL-CEA)	βhCG (FB12)	HER-2/neu (NCL-CBE1)
Melanoma	0/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Sarcoma	0/5	0	0	0	0	1/5	0	0	0	0	3/5	0
Neuroblastoma	0	1/5	0	0	0	0	0	0	0	0	1/5	0
B-cell lymphoma	0/5	0	0	0	0	0	0	5/5 ^b	0	3/5	1/5	0
Small cell lung	1/5	0	0	0	0	0	0	5/7 ^b	0/7	4/7 ^b	4/7	1/7
Breast	5/7 ^b	3/7	1/7	1/7	5/6 ^b	4/6	1/6	5/5 ^b	3/5 ^b	0	1/5	1/5
Metastatic prostate	3/5	4/5 ^b	0	0	1/5	0	0	5/5 ^b	0	4/5 ^b	3/5	1/5
Lung	4/5 ^b	0	1/5	4/5	0	0	4/8	8/8 ^b	0	6/8 ^b	2/8	0/8
Colon	3/8	6/8 ^b	4/8	4/5 ^b	1/8	3/8	4/8	5/5 ^b	0	3/5 ^b	4/5	0
Pancreas	2/5	2/5	0	3/5 ^b	2/5	1/5	0	5/5 ^b	0	5/5 ^b	0	0
Gastric	1/5	0	3/5	2/5	4/5 ^b	1/5	1/5	5/5 ^b	0	2/5	2/5	0
Ovarian	5/5 ^b	2/5	4/5	3/5	0	0	0	5/5 ^b	0	1/5	2/5	0
Endometrial	3/5 ^b	0	2/5	0	1/5	2/5	0	5/5 ^b	0	1/5	2/5	0

* All tumor tissues were stained by ABC immunoperoxidase methods.

^b Median staining intensity was 4+ for ≥80% of cells.

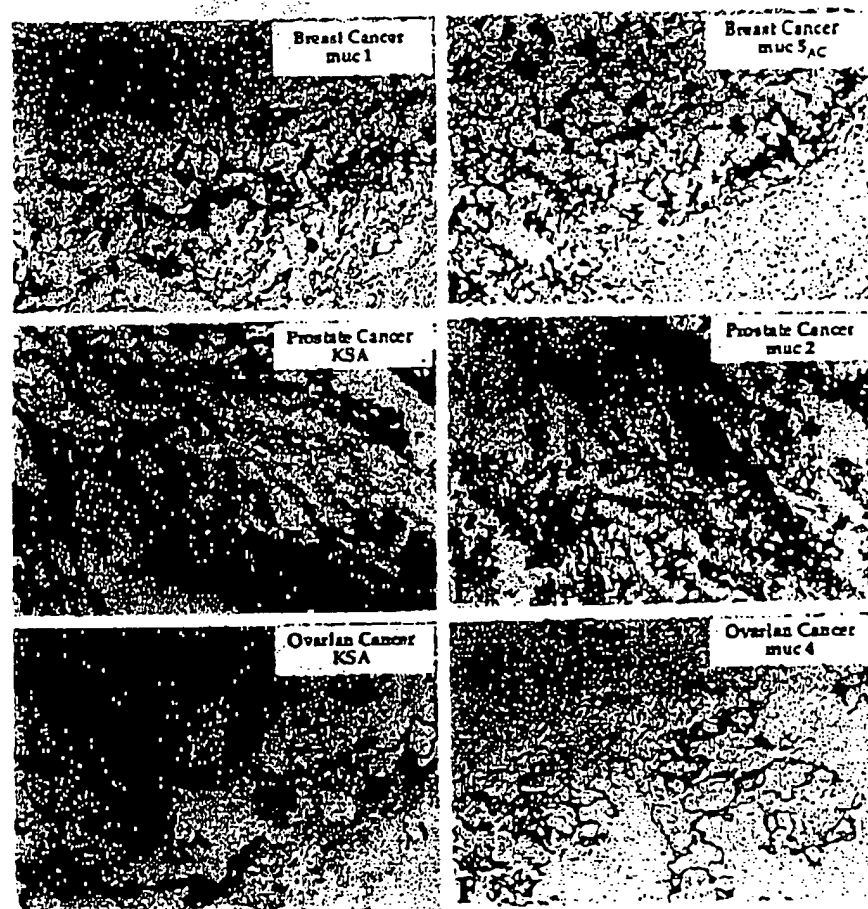
G. Steven Bova (PELICAN Laboratory, Johns Hopkins University, Baltimore, MD). Cryostat sections were cut at 5 μm, dried in air, and fixed with neutral buffered 10% formalin solution (Sigma Co., St. Louis, MO) for 10 min before H&E or immunostaining.

mAb and Immunohistochemistry. The murine mAbs and the antigens they recognize are summarized in Table 1. mAb HMFG-2 was provided by J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom); LDQ10 was provided by F. X. Real (Institut Municipal d'Investigacio Medica, IMIM, Barcelona, Spain); M3.2 and MUC4.275 were provided by V. Apostolopoulos (Austin Research Institute, Victoria, Australia); CLH2, PANH2, and PANH3 were provided by H. Clausen (University of Copenhagen, Copenhagen, Denmark); Cyt351 was provided by W. Heston (Memorial Sloan-Kettering Cancer Center); FB12 was provided by D. Beller (Institut Gustave-Roussy, Villejuif, France); and GA733-2 was provided by D. Herlyn (The Wistar Institute, Philadelphia, PA). mAbs NCL-CEA and NCL-CBE1 were purchased from Vector Laboratories, Inc. (Burlingame, CA).

The ABC immunoperoxidase method was performed as

described previously (33). Briefly, the sections were quenched with 0.1% H₂O₂ in PBS for 15 min, blocked with avidin and biotin reagents (Vector Laboratories) for 10 min each, incubated in 10% serum of horse or goat from which the second antibody was raised, and incubated with various mAbs for 1 h at optimal concentration. The optimal mAb concentration was selected based on the strongest reactivity against the known positive target cells with little or no background against stroma. The concentrations of mAbs used were: FB12, 0.5 μg/ml; Cyt351 and GA733-2, 2 μg/ml; HMFG-2, M3.2, MUC4.275, CLH2, PANH2, and PANH3 (supernatants), between 1:3 and 1:6; LDQ10 and NCL-CBE1 (ascites), 1:15; and NCL-CEA, 1:50. The sections were subsequently incubated with 1:600 biotinylated horse antimouse IgG or 1:300 goat antimouse IgM antibodies (Vector Laboratories) for 40 min and then incubated in 1:50 ABC reagent (Vector Laboratories) for 30 min. Reactions were developed with 0.02% H₂O₂ and 0.1% diaminobenzidine tetrahydrochloride (Sigma) for 2–5 min. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 1–3 min. The immunoreactivities were graded based on the percentage of positive cells and staining intensity above that seen on the negative control: 1+ (weak)

FIG. 1 Expression of protein antigens on breast cancer (A and D), prostate cancer (B and E), and ovarian cancer (C and F). The pattern of staining of cell membrane bound antigens MUC1 (A) and KSA (B and C) is indistinguishable from secreted antigens MUC5_{AC} (D), MUC2 (E), and MUC4 (F). Staining intensity in these sections is graded as follows: A, 2-3+, 80% of tumor cells positive; B, 4+, 100%; C, 4+, 100%; D, 3+, 80%; E, 3+, 80%; F, 3+, 80%. Magnification, $\times 70$.



2+ (moderate), 3+ (strong), and 4+ (very strong or intense). Staining intensities of 2+ or stronger were considered positive (Table 2 and Fig. 1). Known positive and negative control slides were used in each experiment. Results with the several IgM, IgG3, and IgG2 mAbs included in the panel of antibodies tested ruled out nonspecific adherence of particular subclasses of antibodies.

An indirect immunoperoxidase assay was performed as described previously (34) on normal liver, kidney, and stomach samples because these tissues reacted strongly with ABC reagent directly, producing high background. Briefly, the sections were quenched with 0.1% H₂O₂ in PBS for 15 min, blocked with 10% serum, and incubated with mAbs for 1 h at the optimal concentration. The sections were incubated with 1:100 rabbit antimouse immunoglobulin labeled with peroxidase (DAKO Corp., Carpinteria, CA) for 1 h and developed as described for the ABC method.

RESULTS

Reactivity of mAbs with Tumor Tissues. Table 2 summarizes the staining on tumor tissue samples observed with the panel of mAbs. Eighty-two neoplastic tissue specimens representing 13 tumor types were analyzed with each of the 12 antibodies. None of these mAbs reacted consistently with melanoma, neuroblastoma, or B-cell lymphoma specimens, and

only PB12 against β hCG reacted moderately (2+) with some sarcomas. KSA was very strongly expressed (median 4+) on small cell lung cancer and all or most specimens of all of the epithelial cancers. At the other extreme was PSMA, expressed only on primary and metastatic prostate cancer (median, 3+–4+). β hCG was expressed moderately (median, 2+) on some samples of most tumor types, but strong expression (3+) on occasional specimens, such as three of five lung cancer specimens, was also seen. CEA, MUC1, MUC2, and MUC4 were strongly expressed on the majority of some epithelial cancers (median, 3+–4+) but not expressed at all on others. MUC3, MUC5_D, and MUC7 were moderately expressed on the majority of several cancers (median, 2+). MUC5_{AC} was strongly expressed on only breast and gastric cancers. Confidence in all of these results was bolstered by the very strong expression (4+) seen on some specimens with each of these mAbs and complete lack of staining on other specimens. Strong (3+) HER-2/*neu* expression was only seen on one prostate cancer specimen, and the other two positive specimens were 2+, despite using the available ascites at a 1:15 dilution. Consequently, in the absence of a clear positive control, it is not clear whether the lack of staining of more specimens with NCL-CBE1 against HER-2/*neu* was a consequence of low levels of antigen expression, inactive antibody, or problems with the assay. Representative examples of these reactions and our grading

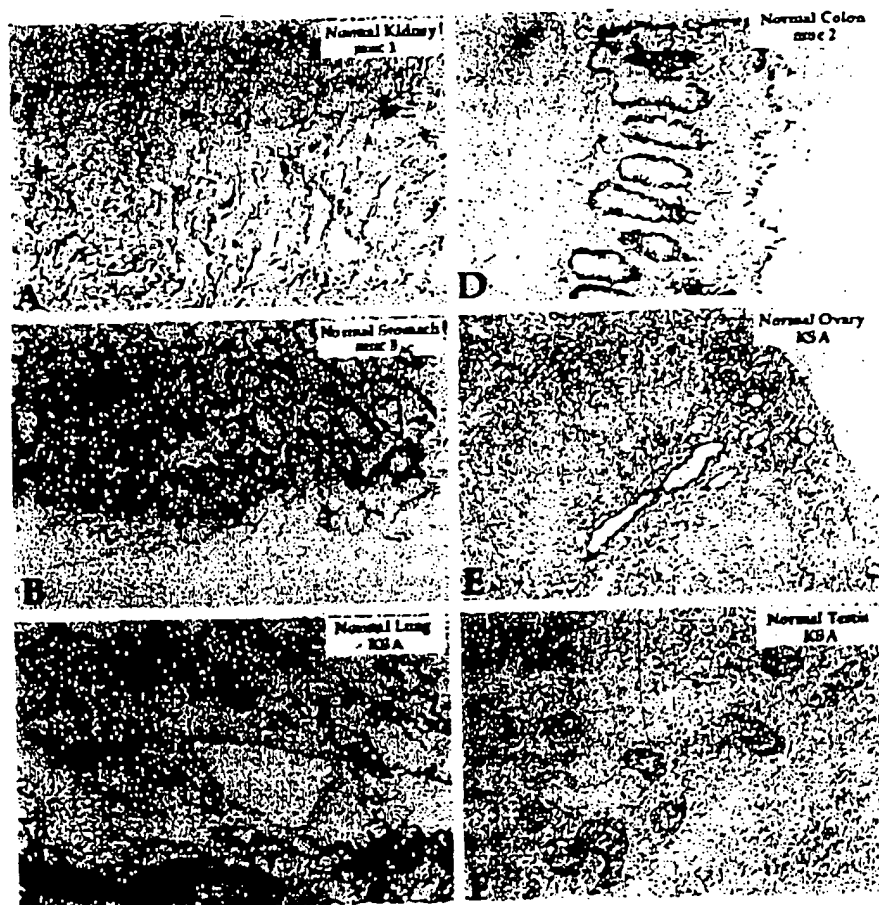
2672 Selection of Tumor Antigens for Immune Attack: Proteins

Table 3 Antigen expression on normal tissues defined by immunohistoLOGY^a

Normal tissue (no.) ^b	Antigen (mAb)											
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (ND-2)	MUC4 (N4-275)	MUC5 _{ac} (CLH2)	MUC5 _{bc} (PANH2)	MUC7 (PANH3)	KSA (GA733-2)	PSMA (CyD51)	CEA (NCL-CEA)	βhCG (FB12)	HER-2/neu (NCL-CBR1)
Spleen (2)	-	-	-	-	-	-	-	-	-	-	-	-
White pulp	-	-	-	-	-	-	-	-	-	-	-	-
Red pulp	-	-	-	-	-	-	-	-	-	-	-	-
Striated muscle (2)	-	-	-	-	-	-	-	-	-	-	-	-
Epithelia												
Lung (2)	2+	-	-	+	-	-	-	3+	-	1+	1+	1+
Breast (2)	1+	-	-	+	-	-	-	3+	-	2+	-	2+
Prostate (6)	±	2+	-	+	-	-	-	4+	3+	3+	3+	3+
Colon (2)	2+	3+	-	3+	-	3+	1+	4+	-	4+	1+	-
Stomach (2)	1+	-	-	-	4+	-	1+	-	-	-	2+	-
Pancreas (2)	2+	2+	2+	-	-	-	1+	4+	-	1+	2+	-
Uterus (2)	1+	-	-	-	-	-	-	3+	-	-	-	-
Ovary (2)	1+	-	-	-	-	-	-	3+	-	-	-	-
Liver (2)	-	-	-	-	-	-	-	1+	-	-	-	-
Kidney (2)	2+	-	-	-	-	-	-	2+	-	-	-	-
Testis (2)	-	-	-	-	-	1+	-	2+	-	-	2+	-
Tissues negative for all 12 antigens												
Brain (3): gray matter, white matter												
Lymph nodes (2)												
Smooth muscle (2)												
Connective tissue (2 each): lung, breast, prostate, colon, stomach, pancreas, uterus, ovary, liver, and kidney												

^a All tissues were stained by ABC immunoperoxidase method, except stomach, liver, and kidney, which were stained by the indirect immunoperoxidase method.^b The numbers in parentheses indicate the number of different specimens tested.^c Histocytes in the red pulp were predominantly stained.^d Seminiferous tubules were stained.

Fig. 2 Expression of protein antigens on normal tissues. Epithelial cells at secretory borders were stained in kidney (A) with mAb HMFG2 against MUC1 (2+); in stomach (B) with mAb CLH2 against MUC5_{AC} (4+); in lung (C), ovary (E), and testis (F) with mAb GA733-2 against KSA (4+, 2+, and 3+, respectively) and in colon (D) with mAb LDQ10 against MUC2 (3+). Magnification, $\times 70$.



of percentage positive tumor cells and staining intensity are shown in Fig. 1. Staining of stroma with all 12 of these mAbs was uniformly negative.

Reactivity of mAbs with Normal Tissues. Table 3 summarizes the immunoreactivity on normal tissue samples observed with the panel of mAbs. MUC1 was weakly distributed on the epithelia of all of the tested organs, except liver. MUC2 was observed on the epithelia of prostate, colon, and pancreas. MUC3 was only detected on epithelia of pancreas. MUC4 was expressed on epithelia of colon and prostate (weakly). MUC5_{AC} was very strongly expressed in stomach epithelium. MUC7 and HER-2/*neu* were not expressed on any normal tissues, and MUC5_B was only detected on normal colon epithelium and weakly in the testis. β hCG was detected in epithelia of prostate, stomach, and pancreas and weakly in colon and lung, and it was detected in the testis. PSMA was only detected on prostate epithelia. KSA was strongly expressed on the epithelia of all of the tested organs except stomach and liver and moderately expressed on seminiferous tubules of testis. CEA was strongly expressed in the epithelia of prostate and colon and weakly in lung, uterus and breast. The pattern of expression of each of these antigens on normal epithelia was mainly luminal, with evident polarity. Luminal cells stained most intensively at luminal borders. In addition, CEA was detected in histiocytes in the red pulp of the spleen, an expected consequence not of CEA

expression but of the mAb used, NCL-CEA, which cross-reacts with nonspecific cross-reacting antigen on histiocytes (35). Examples of the staining patterns on normal tissues with these mAbs are shown in Fig. 2. Once again, staining of stroma was uniformly negative.

DISCUSSION

One of the striking features of our two previous reports was the clear separation between the carbohydrate antigens expressed by tumors of neuroectodermal origin and the carbohydrate antigens expressed by tumors of epithelial origin (1, 2). This is also the case for the protein antigens studied here. None of the seven mucins were expressed on more than one of the specimens of the five nonepithelial origin cancers, but these mucins were widely and densely expressed on a variety of epithelial cancers. The same applies for the other glycoproteins, except that all small cell lung cancers expressed KSA very strongly and some expressed CEA, and some sarcomas expressed moderate amounts of β hCG. On this basis, melanomas, sarcomas, neuroblastomas, and B-cell lymphomas are quite distinct from the eight epithelial cancers tested. Small cell lung cancer, not surprisingly, is intermediary, with some characteristics of each group.

This study differs from previous reports on the distribution

2674 Selection of Tumor Antigens for Immune Attack: Proteins

of these antigens in several ways. Our focus was entirely on potential targets for immunotherapy and, especially, on antibody-mediated immunotherapy. We have compared the expression of 12 antigens rather than 1 to 3 and explored a wide variety of malignant and normal tissues rather than a few. On the other hand, we tested only five to eight specimens in most cases, and because this was part of a larger study looking at expression of glycolipid antigens as well, specimens were fixed with 10% formalin, which may not be ideal for some protein antigens. However, to the extent that others have studied the expression of these antigens on these cancers, our study is largely in agreement. MUC1 has long been known to be expressed by many normal epithelial tissues and by many or most cancers of breast, ovary, pancreas, prostate, and colon origin (21-23, 26). We concur and add to this list endometrial and non-small cell lung cancer. MUC2 has been previously identified in most colon cancers as well as cancers of the stomach, pancreas, breast, and, recently, prostate (22, 24, 36). We concur, except that we found no evidence of MUC2 in the five gastric cancer specimens we tested. The previously described more restricted expression of MUC2 than MUC1 on normal tissues (22, 24, 36, 37), with MUC2 detected in the GI tract and, recently, the prostate (36) but not most other sites, was also our finding. MUC3 was previously detected on the majority of colon, ovarian, and gastric cancers (20, 24), in agreement with our findings, but also in the GI tract but not the pancreas, which is the reverse of our findings. MUC5_{AC} has previously been detected in the majority of gastric cancers and in normal stomach, as we found, and we add to this strong expression in most breast cancers (22, 27). MUC5_B has been described to be strongly expressed on some colorectal carcinomas and normal colon (22), as we found. We add to this moderate expression of MUC5_B on the majority of breast cancers. Our study breaks little new ground on the distribution of KSA (38) and PSMA (4, 28, 36), except that we were not prepared for the intensity and uniformity of KSA expression on all epithelial cancers tested (and normal epithelial tissues), and we have extended the number of different normal tissues and nonprostate cancers that are negative for PSMA by immunohistology. Likewise, we confirm the strong expression of CEA on most breast, lung, and GI malignancies and the corresponding normal tissues as described previously (35, 39, 40). β hCG mRNA has been described to be strongly expressed in 61% of bladder cancers (which we did not test) and to be moderately expressed in 46% of breast cancers and 20% of prostate cancers (19), which agrees with our findings. We add to this moderate expression in a small proportion of several other cancers and the majority of sarcomas and cancers of the lung and pancreas, as well as a variety of normal tissues.

A benefit of testing many different types of cancers with a broad range of mAbs is that it permits selection of the several antigens most suitable as targets for immune attack against each cancer. Expression on normal tissues is, of course, a consideration in this selection, but expression at the secretory border of epithelial tissues does not appear to be a problem (as discussed at greater length in part I of this series; Ref. 1). Antigens expressed at epithelial secretory borders induce neither immunological tolerance nor detectable autoimmunity once antibodies are administered or induced against them. Consequently, if strong expression on $\geq 80\%$ of tumor cells of 60% or more of

Table 4 Protein targets for antibody-mediated immunotherapy^a

Cancer	Antigens ^b
Melanoma	None
Sarcoma	(β hCG)
Neuroblastoma	None
B-cell lymphoma	None
Small cell lung cancer	KSA
Breast	MUC1, MUC5 _{AC} , (KSA), (CEA)
Prostate	MUC2, KSA, (PSMA)
Lung	MUC1, CEA, KSA, (MUC4), (β hCG)
Colon	MUC2, CEA, KSA, (MUC4)
Pancreas	KSA, (MUC4), (CEA), (β hCG)
Gastric	MUC5 _{AC} , CEA, KSA, (MUC3)
Ovarian	MUC1, KSA, (MUC3)
Endometrial	KSA, (MUC1)

^a Targets selected from the 12 antigens tested in this study.

^b Antigens expressed intensely (4+) on $\geq 80\%$ of tumor cells in $\geq 70\%$ of specimens. Antigens in parentheses were expressed on $\geq 80\%$ of tumor cells strongly (3+) on at least 50% or moderately (2+) on at least 60% of specimens.

the cancer specimens tested but not on immune accessible tissues are used as selection criteria, the antigens selected as targets for each cancer are shown in Table 4. The results summarized in Table 4 for protein antigens plus the corresponding tables for ganglioside and carbohydrate antigens in parts I and II of this series (1, 2) provide the basis for selection of multiple antigens as targets for antibody-mediated immune attack against these cancers.

ACKNOWLEDGMENTS

We thank Drs. J. Taylor-Papadimitriou (mAb HMFG-2), F. X. Real (mAb LDQ10), V. Apostolopoulos (mAbs M3.2 and MUC4.275), H. Clausen (mAbs CLH2, PANH2, and PANH3), W. Heston (mAb Cyt351), D. Bellet (mAb FB12), and D. Herlyn (mAb GA733-2) for providing the indicated mAbs and Dr. G. S. Buva for providing metastatic prostate cancer specimens.

REFERENCES

- Zhang, S., Cordon-Cardo, C., Zhang, H. S., Reuter, V. E., Adluri, S., Hamilton, W. B., Lloyd, K. O., and Livingston, P. O. Selection of tumor antigens as targets for immune attack using immunohistochemistry. I. Focus on gangliosides. *Int. J. Cancer*, 73: 42-49, 1997.
- Zhang, S., Zhang, H. S., Cordon-Cardo, C., Reuter, V. E., Singhal, A. K., Lloyd, K. O., and Livingston, P. O. Selection of tumor antigens as targets for immune attack using immunohistochemistry. II. Blood group-related antigens. *Int. J. Cancer*, 73: 50-56, 1997.
- Robbins-Roth, C. Cancer vaccines: are we finally on the right track? *Bioventure View*, 12: 2-10, 1997.
- Fair, W. R., Israeli, R. S., and Heston, W. D. W. Prostate-specific membrane antigen. *Prostate*, 32: 140-148, 1997.
- Mclaughlin, J. P., Schlom, J., Kantur, J. A., and Greiner, J. W. Improved immunotherapy of a recombinant carcinoembryonic antigen vaccine when given in combination with interleukin-2. *Cancer Res.*, 56: 2361-2367, 1996.
- Talwar, G. P., Singh, O., Pal, R., and Chatterjee, N. Vaccines for control of fertility and hormone dependent cancers. *Int. J. Immunopharmacol.*, 14: 511-514, 1992.
- Disis, M. L., Shiot, F. M., and Cheever, M. A. Human Her-2/neu protein immunization circumvents tolerance to rat neu: a vaccine strategy for "self" tumour antigens. *Immunology*, 93: 192-199, 1998.

8. Coussens, L., Yang-Feng, T. L., Chen, Y. L. E., Gray, A., McGrath, J., Seeburg, P. H., Liberman, T. A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to *egf* receptor shares chromosomal location with *neu* oncogene. *Science* (Washington DC), 230: 1132-1139, 1985.
9. Israeli, R. S., Powell, C. T., Fair, W. R., and Heston, W. D. W. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, 53: 227-230, 1993.
10. Gendler, S., Lancaster, C., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.-N., and Wilson, D. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J. Biol. Chem.*, 265: 15286-15293, 1990.
11. Jessup, J. M., and Thomas, P. Carcinoembryonic antigen: function in metastasis by human colorectal carcinoma. *Cancer Metastasis Rev.*, 8: 263-280, 1989.
12. Perez, M. S., and Walker, L. E. Isolation and characterization of a cDNA encoding the KS1/4 epithelial carcinoma marker 1. *J. Immunol.*, 142: 3662-3667, 1989.
13. Bohck, L. A., Tsai, H., Biesbrock, A. R., and Levine, M. J. Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J. Biol. Chem.*, 266: 20563-20569, 1993.
14. Dufosse, J., Porchet, N., Audie, J.-P., Duperat, V. G., Laine, A., Van Seuningen, I., Marrakchi, S., Degand, P., and Aubert, J.-P. Degenerate 87-base-pair tandem repeats create hydrophilic/hydrophobic alternating domains in human peptides mapped to 11p15. *J. Biochem.*, 293: 329-337, 1993.
15. Duperat, V. G., Audie, J.-P., Dehaillet, V., Laine, A., Buisine, M.-P., Gallieue-Zouitina, S., Pigny, P., Degand, P., Aubert, J.-P., and Porchet, N. Biochemical characterization of the human mucin gene *MUC5AC*: a consensus cysteine-rich domain for 11p15 mucin genes. *Biochem. J.*, 305: 211-219, 1995.
16. Gum, J. R., Byrd, J. C., Hicks, J. W., Toribara, N. W., Lamport, D. T. A., and Kim, Y.-S. Molecular cloning of human intestinal mucin cDNAs: sequence analysis and evidence for genetic polymorphism. *J. Biol. Chem.*, 264: 6480-6487, 1989.
17. Gum, J. R., Hicks, J. W., Swallow, D. W., Legace, R. L., Byrd, J., Lamport, D. T., Siddiqui, B., and Kim, Y.-S. Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem. Biophys. Res. Commun.*, 171: 407-415, 1990.
18. Porchet, N., Nguyen, V. C., Dufosse, J., Audie, J. P., Guyonnet-Duperat, V., Gross, M. S., Denis, C., Degand, P., Bernheim, A., and Aubert, J. P. Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem. Biophys. Res. Commun.*, 175: 414-422, 1991.
19. Bellet, D., Lazar, V., Bieche, I., Paradis, V., Giovannardi, Y., Paterlini, P., Lidereau, R., Bedossa, P., Bidart, J.-M., and Vidaud, M. Malignant transformation of non-trophoblastic cells is associated with the expression of chorionic gonadotropin B genes normally transcribed in trophoblastic cells. *Cancer Res.*, 57: 516-523, 1997.
20. Apostolopoulos, V., Xing, P.-X., and McKenzie, I. P. C. Anti-peptide monoclonal antibodies to intestinal mucin 3. *J. Gastro. Hepatol.*, 10: 555-561, 1995.
21. Byrd, J. C., Hu, J. J. L., Lamport, D. T. A., Ho, S. B., Siddiqui, B., Huang, J., Yan, and Kim, Y. S. Relationship of pancreatic cancer apomucin to mammary and intestinal apomucins. *Cancer Res.*, 51: 1026-1033, 1991.
22. Carrato, C., Balague, C., De Bolos, C., Gonzalez, E., Gambus, G., Plana, J., Perini, J. M., Andreu, D., and Real, F. X. Differential apomucin expression in normal and neoplastic human gastrointestinal tissues. *Gastroenterology*, 107: 160-172, 1994.
23. Dienhart, D. G., Schmelter, R. F., Lear, J. L., Miller, G. J., Glenn, S. D., Bloodow, D. C., Kasliwal, R., Moran, P., Seligman, P., Murphy, J. R., Kortright, K., and Bunn, P. A., Jr. Imaging of non-small cell lung cancers with a monoclonal antibody, KC-4G3, which recognizes a human milk fat globule antigen. *Cancer Res.*, 50: 7068-7076, 1990.
24. Ho, H. B., Nichols, G. A., Lyfeg, C., Yan, P. S., Cherwitz, D. I., Gum, E. T., Dahiya, R., and Kim, Y. S. Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res.*, 53: 641-651, 1993.
25. Hollingsworth, M. A., Strawhecker, J. M., Calhrey, T. C., and Mack, D. R. Expression of *muc1*, *muc2*, *muc3* AND *muc4* mucin mRNAs in human pancreatic and intestinal tumor cell lines. *Int. J. Cancer*, 57: 198-203, 1994.
26. Ichige, K., Perey, L., Vogel, C. A., Buchegger, F., and Kufe, D. Expression of the DF3-P epitope in human ovarian carcinomas. *Clin. Cancer Res.*, 1: 565-571, 1995.
27. Reis, C. A., David, L., Nielsen, P. A., Clausen, H., Mirgorodskaya, K., Ruepfert, P., and Sobrinho-Simoes, M. Immunohistochemical study of MUC5AC expression in human gastric carcinomas using a novel monoclonal antibody. *Int. J. Cancer*, 74: 112-121, 1997.
28. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D. W., and Cordon-Cardo, C. Prostate specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.*, 3: 81-85, 1997.
29. Yu, C.-J., Yang, P.-C., Shun, C.-T., Lee, Y.-C., Kuo, S.-H., and Luh, K.-T. Overexpression of *MUC5* genes is associated with early post-operative metastasis in non-small-cell lung cancer. *Int. J. Cancer*, 69: 457-465, 1996.
30. Livingston, P. O., and Ragupathi, G. Carbohydrate vaccines that induce antibodies against cancer. Part II: Previous experience and future plans. *Cancer Immunol. Immunother.*, 45: 10-19, 1997.
31. Livingston, P. O., Wong, G. Y. C., Aduri, S., Tao, Y., Padavan, M., Parente, R., Hanlon, C. M. J., Helling, F., Ritter, G., Oettingen, H. F., and Old, L. J. Improved survival in AJCC stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. *J. Clin. Oncol.*, 12: 1036-1044, 1994.
32. MacLean, G. D., Reddish, M. A., Koganty, R. R., and Longenecker, B. M. Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic sTn vaccine. *J. Immunol.*, 159: 59-68, 1996.
33. Hsu, S. M., Rain, L., and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29: 577-580, 1981.
34. Dippold, W. G., Lloyd, K. O., Li, L. T., Ikeda, H., Oettingen, H. F., and Old, L. J. Cell-surface antigens of human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, 77: 6114-6118, 1980.
35. Thompson, J. A., Grunert, F., and Zimmerman, W. Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J. Clin. Lab. Anal.*, 5: 344-366, 1991.
36. Zhang, S., Zhang, H. S., Reuter, V. E., Lloyd, K. O., Scher, H., and Livingston, P. O. Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. *Clin. Cancer Res.*, 4: 295-302, 1998.
37. Gambus, G., Bolos, C. D., Andreu, D., Franci, C., Egea, G., and Real, F. X. Detection of the MUC2 apomucin tandem repeat with a mouse monoclonal antibody. *Gastroenterology*, 104: 93-102, 1993.
38. Gottlinger, H. G., Funke, I., Johnson, J. P., Gokel, J. M. M., and Riehmuller, G. The epithelial cell surface antigen 17-1A, a target for antibody-mediated tumor therapy: its biochemical nature, tissue distribution and recognition by different monoclonal antibodies. *Int. J. Cancer*, 38: 47-53, 1986.
39. Hayes, D. F., Zurawski, V. R., and Kufe, D. W. Comparison of circulating CA15- and carcinoembryonic antigen levels in patients with breast cancer. *J. Clin. Oncol.*, 4: 1542-1550, 1986.
40. Shimada, S., Ogawa, M., Schlom, J., and Greiner, J. W. Identification of a novel tumor-associated M_r 110,000 gene product in human gastric carcinoma cells that is immunologically related to carcinoembryonic antigen. *Cancer Res.*, 51: 5694-5703, 1991.
41. Taylor-Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R., and Bodmer, W. F. Monoclonal antibodies to epithelium

2676 Selection of Tumor Antigens for Immune Attack: Proteins

specific components of the milk fat globule membrane: production and reactions with cells in culture. *Int. J. Cancer*, 28: 17-21, 1981.

42. Gendler, S. J., Spicer, A. P., Lalani, E.-N., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Beshell, M., and Taylor-Papadimitriou, J. Structure and biology of a carcinoma associated mucin, MUC1. *Am. Rev. Respir. Dis.* 144 (Suppl.): s42-s47, 1991.

43. Xing, P. X., Prenzioska, J., Apostolopoulos, V., Karkaloutsos, J., and McKenzie, I. F. C. Monoclonal antibodies to MUC4: peptide react with lung cancer. *Int. J. Oncol.* 11: 289-295, 1997.

44. Nielsen, P. A., Mandel, U., Therkildsen, M. H., and Clausen, H. Differential expression of human high molecular weight salivary mucin (MG1) and low molecular weight salivary mucin (MG2). *J. Dental Res.* 75: 1820-1826, 1996.

45. Nielsen, P. A., Bennett, E. P., Wandall, H. H., Therkildsen, M., Hannibal, J., and Clausen, H. Identification of a major human high

molecular weight salivary mucin (MG1) as tracheobronchial mucin MUC5B. *Glycobiology*, 7: 413-419, 1997.

46. Heston, D. W. Biologic implications for prostatic function following identification of prostate-specific membrane antigen as a novel folate hydrolase/neurocarboxypeptidase. In: R. K. Naz (ed.), *Prostate: Basic and Clinical Aspects*, pp. 267-294. New York: CRC Press, 1997.

47. Hertlyn, D., Hertlyn, M., Ross, A. H., Ernst, C., Atkinson, B., and Koprowski, H. Efficient selection of human tumor growth-inhibiting monoclonal antibodies. *J. Immunol. Methods*, 73: 157-167, 1984.

48. Bellet, D. H., Ozturk, M., Bidart, J. M., Bouhuon, C. J., and Wands, J. R. Sensitive and specific assay for human chorionic gonadotropin (hCG) based on anti-peptide and anti-hCG monoclonal antibodies: construction and clinical implications. *J. Clin. Endocrinol. Metab.* 63: 1319-1326, 1986.



ARTICLE

Mucins (MUC1 and MUC3) of Gastrointestinal and Breast Epithelia Reveal Different and Heterogeneous Tumor-associated Aberrations in Glycosylation

Yi Cao, Dietmar Blohm, B. Michael Ghadimi, Peter Stosiek, Pei-Xiang Xing, and Uwe Karsten

Max Delbrück Center for Molecular Medicine, Berlin-Buch (YC,UK), Biotechnology and Molecular Genetics, University of Bremen, Bremen (DB), Robert Rössle Clinic, Rudolf Virchow Klinikum, Humboldt University, Berlin (BMG), Institute of Pathology, Carl Thiem Klinikum, Cottbus (PS), Germany, and Austin Research Institute, Heidelberg, Australia (PXX)

SUMMARY In a comprehensive study, we examined the expression of the membrane and secretory mucins MUC1 and MUC3, respectively, in normal and neoplastic gastrointestinal and breast epithelia before and after specific alterations of their glycan structures by neuraminidase, α -fucosidase, or carbohydrate-specific periodate oxidation. MUC1 mRNA was also identified in normal colorectal tissues by in situ hybridization. The data revealed that normal colorectal epithelia express both MUC1 mRNA and protein, which were detectable after periodate oxidation with all tested MUC1-specific antibodies. During tumorigenesis in the colon, MUC1 became recognizable without periodate treatment concomitantly with highly dysplastic lesions and the malignant state. In the breast, in which MUC1 is detectable with most antibodies in normal epithelium as well as in carcinomas, staining could be enhanced by pretreatment with periodate and casually by enzyme treatments. MUC3 was detectable in normal and neoplastic colorectal tissues and was more intensely stained after periodate oxidation. It was absent in normal breast even after pretreatment but was expressed in seven of 20 breast carcinomas. Therefore, incomplete glycosylation, abnormal distribution, and ectopic expression of mucins are characteristics of malignancy. Periodate oxidation may be widely applicable to immunohistochemistry for examining changes in glycosylation and for detecting antigens masked by glycans.

(J Histochem Cytochem 45:1547-1557, 1997)

KEY WORDS

MUC1
MUC3
immunohistochemistry
in situ hybridization
carbohydrate
periodate oxidation
gastrointestinal tract
breast
tumor

MUCINS are a family of highly glycosylated, high molecular weight glycoproteins present on the surface of many glandular epithelial cells and in their secretions (Devine and McKenzie 1992; Strous and Dekker 1992; Jass and Robertson 1994). They consist of a nonglobular, threadlike protein backbone (apomucin) and many predominantly O-linked glycans. The best-known function of mucins is to provide a barrier between the luminal membranes of epithelial cells and their environment. In the digestive tract, this barrier is most important because it protects the epithelial cells from the action of degradative enzymes, particularly proteases. Mucins can be subdivided into two types,

secretory and membrane-bound mucins. Secretory mucins provide to a large part the viscous gel that covers most mucosal surfaces of respiratory, gastrointestinal, and reproductive tracts. Membrane-bound mucins are major components of the apical surface of epithelial cells, which contain a hydrophobic stretch of amino acid residues anchoring the long filamentous molecules in the plasma membrane. Several distinct cDNAs of mucin core proteins have been isolated that are encoded by the muc1-8 genes. MUC1 (previously labeled PUM, PEM, MAM-6, PAS-O, EMA, NPG, HMFGP), the muc1 gene product, is a membrane-bound mucin (Patton et al. 1995). Its protein backbone consists of 20 amino acid residues repeated manyfold in tandem, a transmembrane sequence, and a 69 amino acid-long cytoplasmic tail. The tandem repeats are rich in serine and threonine as potential O-glycosylation sites. MUC1 has been described to be expressed in normal breast, pancreas, and other glan-

Correspondence to: Uwe Karsten, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str.10, D 13122 Berlin-Buch, Germany.

Received for publication March 3, 1997; accepted June 19, 1997 (7A4255).

1548

dular epithelial tissues (Zotter et al. 1988; Girling et al. 1989; Ho et al. 1993; Patton et al. 1995). MUC3 is a secretory mucin; its central region is composed of an extended region of tandem repeat sequences of 17 amino acids each. It is present in normal gastrointestinal secretions and epithelia and in some tumors (Gum et al. 1990). Overexpression of mucins and mucin-associated glycotopes on the surface of tumor cells provides valuable tumor markers for clinical diagnosis and targets for immunotherapy (Apostolopoulos and McKenzie 1994; Taylor-Papadimitriou and Epenetos 1994; Graham et al. 1996). In addition, the increased expression of MUC1 contributes to the malignant phenotype (Hilkens et al. 1995). However, several important questions remain to be answered. (a) Do normal intestinal epithelial cells express MUC1? This is a question at issue. Most reports have suggested that normal intestinal epithelial cells do not or only sparsely react with monoclonal antibodies (MAbs) to MUC1 (Zotter et al. 1988; Girling et al. 1989; Ho et al. 1993, 1996; Jass and Robertson 1994), although a few authors have claimed that normal adult intestine could be stained by their MUC1 MAbs (Carrato et al. 1994; Hilkens et al. 1995). Recent studies have reported that these cells transcribe the *mucl* gene into mRNA (Ogata et al. 1992; Ho et al. 1993; Nakamori et al. 1994). (b) Why is the MUC1 apomucin detectable in colorectal carcinomas, but apparently absent in normal intestinal mucosae? (c) Do the glycan side chains interfere with the binding of apomucin-specific MAbs to intestinal MUC1? In contrast to the breast/milk mucin, the glycans of intestinal MUC1 are only incompletely known (Podolsky 1985).

In this study, serial sections from various gastrointestinal (normal stomach, ileum, and colon; colorectal adenomas and carcinomas) and mammary tissues (normal breast and mammary carcinomas) were used to examine the presence of MUC1 mRNA in the different cell types of normal gastrointestinal by *in situ* hybridization and the expression of MUC1 and MUC3 proteins, the latter as an example of a secretory mucin, by immunohistochemistry with MAbs to peptide epitopes before and after glycan-inhibiting measures such as mild periodic acid oxidation, α -fucosidase, or neuraminidase incubation. The results show that MUC1 is indeed expressed in normal colorectal mucosa but is inaccessible to anti-MUC1 peptide-recognizing MAbs. Epitope masking by glycans can be overcome by pretreatment of the tissue sections with periodate.

Materials and Methods

Normal Adult Human Tissues and Tumor Specimens

Two stomach (including corpus and pyloric antrum), one ileum, and four colon specimens were obtained at autopsy from individuals without colon disease. One ileum and 15

Cao, Blohm, G. Jilmi, Stosiek, Xing, Karsten

colon tissue samples were taken at surgery or colonoscopy from apparently normal parts of the colon wall distant from the tumor. All mucosae showed normal histomorphological and cytomorphological findings. Twenty-three cases of colorectal adenomas and 22 carcinomas were obtained at surgery or colonoscopy. Adenomatous polyps were classified as to histological type and grade of dysplasia according to the criteria of Konishi and Morson (1982). Classification of primary carcinomas was done with hematoxylin-eosin-stained sections according to World Health Organization (WHO) recommendations (Jass and Sobin 1989).

Twenty mammary carcinomas and 10 normal mammary tissues from patients with cancer were derived from surgical specimens. The tumors were classified according to the criteria of the WHO (1982) and the Bloom-Richardson system (Elston and Ellis 1991). The ABO blood group type and the secretor status of the donors were also documented for all mammary tissues.

Two of the normal stomach, ileum, and colon samples and all mammary samples were fixed in 10% buffered formalin and embedded in paraffin. All other tissues were immediately frozen and stored at -80°C .

In Situ Hybridization

RNA Probe. The MUC1 cDNA probe, *pum24P* (Swallow et al. 1987), was sequenced. A 171-bp fragment from the tandem repeat of the MUC1 cDNA is similar to that of other described MUC1 cDNAs (Siddiqui et al. 1988; Hareuveni et al. 1990). Digoxigenin (DIG)-labeled RNA transcripts were synthesized from the pBluescript plasmids linearized by Hind III and BamH I with T3 and T7 polymerases to generate anti-sense and sense probes, respectively. Unlabeled anti-sense was also synthesized.

***In Situ* Hybridization (ISH).** ISH was performed as described (Schaeren-Wiemers and Gerfin-Moser 1993) in two samples each of normal stomach, ileum, and colon. Deparaffinized sections or formalin-fixed cryosections were digested with proteinase K (Boehringer, Mannheim, Germany) [10 $\mu\text{g}/\text{ml}$ in PBS, 20 min at room temperature (RT)]. After three rinses in PBS, sections were treated with 0.5% and 1% triethanolamine, pH 8.0, and 0.25% acetic anhydride for 5 min. Prehybridization was done with hybridization buffer [50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, pH 7.0 (5 \times SSC), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.25 mg/ml tRNA, 1.25 mg/ml herring sperm DNA, 4 mM EDTA] at 45°C for 3 hr. The hybridization mixture was prepared by adding 1 μl DIG-RNA probe per 50 μl hybridization buffer, heated for 5 min at 85°C to denature the probe, and then chilled on ice. Fifty μl of this hybridization mixture was added per slide, which was then covered with a coverslip and sealed with rubber cement. Hybridization was done overnight at 72°C. Posthybridization washings were successively performed in SSC solutions of decreasing concentrations (5 \times SSC, 50% formamide, 10 min at 50°C; 2 \times SSC, 25% formamide, three times for 10 min at 42°C and twice for 30 min at 42°C; 0.2 \times SSC, twice for 5 min at RT). Thereafter, the sections were rinsed three times with PBS and incubated with Fab fragments from anti-DIG antibody conjugated to alkaline phosphatase (Boehringer; 1:500) for 1 hr at RT. After three wash-

MUC1 and MUC3 in Colon and Breast Tissues

ing steps, the color development was performed with the Fast Red Substrate System (DAKO; Hamburg, Germany). Controls for in situ hybridization consisted of (a) DIG-labeled sense RNA probe, (b) competition with a 20-fold excess of unlabeled anti-sense RNA in the presence of DIG-labeled anti-sense RNA, (c) pretreatment of sections with ribonuclease, and (d) hybridization mixture without RNA probe.

Immunohistochemistry

Monoclonal Antibodies. The antibody A76-A/C7 (IgG₁) was developed in this laboratory (Cao et al. 1997a,b). It recognizes the peptide epitope APDTRP of the MUC1 tandem repeat (O. Nilsson et al., communicated at the ISOBM TD-4 Workshop, San Diego, CA, November 17-22, 1996). The anti-MUC1 antibody HMFG-2 (IgG₁) recognizing the epitope DTR (Burchell et al. 1989) was purchased from Dianova (Hamburg, Germany). SM3 (IgG₁) specific for the MUC1 epitope PDTRP (Burchell et al. 1989) was kindly donated by Dr. G. Pecher (MDC). M3.2 (anti-MUC3, IgG₂) (Apostolopoulos et al. 1995) recognizes a peptide epitope of MUC3. MAbs to group A and B antigens were purchased from DAKO. MAb A46-B/B10 (IgM), specific for the H Type 2 antigen (Karsten et al. 1988), and A78-G/A7 (IgM), specific for the Thomsen-Friedenreich antigen (Karsten et al. 1995), have been described.

Tissue Staining. Staining of tissue sections was performed by the avidin-biotin-peroxidase complex (ABC) method with a commercial kit (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) as follows. Paraffin sections (4 μ m) were deparaffinized in xylene and rehydrated through a graded ethanol series. Frozen sections (4 μ m) were air-dried at RT and fixed with 10% formalin in PBS for 15 min at RT. Endogenous peroxidase activity was eliminated by treatment with 3% H₂O₂ in PBS for 30 min at RT. Non-specific binding sites were blocked with normal rabbit serum. After washing with PBS, sections were incubated with MAbs in appropriate dilutions for 1 hr at RT. The thoroughly washed sections were treated with biotinylated anti-mouse immunoglobulin antiserum for 30 min at RT and thereafter with the ABC complex. Color development during incubation with the peroxidase substrate (diaminobenzidine) was controlled under the microscope. Counterstaining was done with hematoxylin. Negative controls were incubated with comparable dilutions of IgM or IgG paraproteins from mouse plasmacytomas (Sigma; Daisenhofen, Germany) instead of the MAb.

Partial Deglycosylation. One of the following pretreatments was performed on the sections before incubation with anti-MUC1 or MUC3 MAbs.

Neuraminidase. Sections were incubated with neuraminidase from *Vibrio cholerae* (Serva; Heidelberg, Germany) at a concentration of 0.02 U/ml in PBS containing 0.01 M Ca⁺⁺ for 1 hr at RT to remove sialic acid residues. After enzyme treatment, sections were rinsed for 5 min in PBS. Positive control of neuraminidase action was achieved by staining of erythrocytes and endothelial cells of a tissue section by MAb A78-G/A7 (anti-Thomsen-Friedenreich antigen) after the enzyme treatment.

α -Fucosidase. Sections were incubated for 24 hr at 37°C with α -fucosidase (bovine kidney, Boehringer; 0.4 U/ml in acetate buffer, 0.2 M, pH 5). Then they were rinsed for 5 min in PBS. Loss of staining of erythrocytes and endothelial cells in tissue sections from individuals with blood group O with the anti-H₂ MAb A46-B/B10 served as control of enzyme action.

Periodate Oxidation. Sections were treated as described (Bara et al. 1992). Briefly, they were incubated for 30 min at RT with 20 mM periodic acid in acetate buffer, 0.05 M, pH 5. After three rinses in PBS, the sections were treated with 1% glycine for 30 min, followed by three rinses in PBS. This treatment destroyed glycotopes containing galactose, N-acetylgalactosamine, or fucose in cell preparations or tissue sections, but did not damage peptide epitopes (Cao et al. 1997a).

Blood Groups and Secretor Status. Bara et al. (1993) reported that in normal gastric mucosa the immunodetection of MUC1 in secretors was different from that in nonsecretors. We therefore collected blood group and secretor data on patients. In breast cancer patients, ABO data were available from the clinical records. The secretor status was determined from saliva according to standard procedures. Because in the gastrointestinal and normal breast tissue donors neither their blood group nor their secretor status was known to us, we applied anti-H₂, anti-A, and anti-B MAbs to some sections, and examined normal epithelial cells, red blood cells, and endothelia for reactivity. According to the results, secretor status and blood group of the donors were determined.

Scoring. For normal mucosa, the percentage of positive crypts was counted, whereas in carcinomas the percentage of positive cells in several optical fields (12.5 \times lens) was estimated. For further details, see legends to Figures 2-4 and 6.

Results

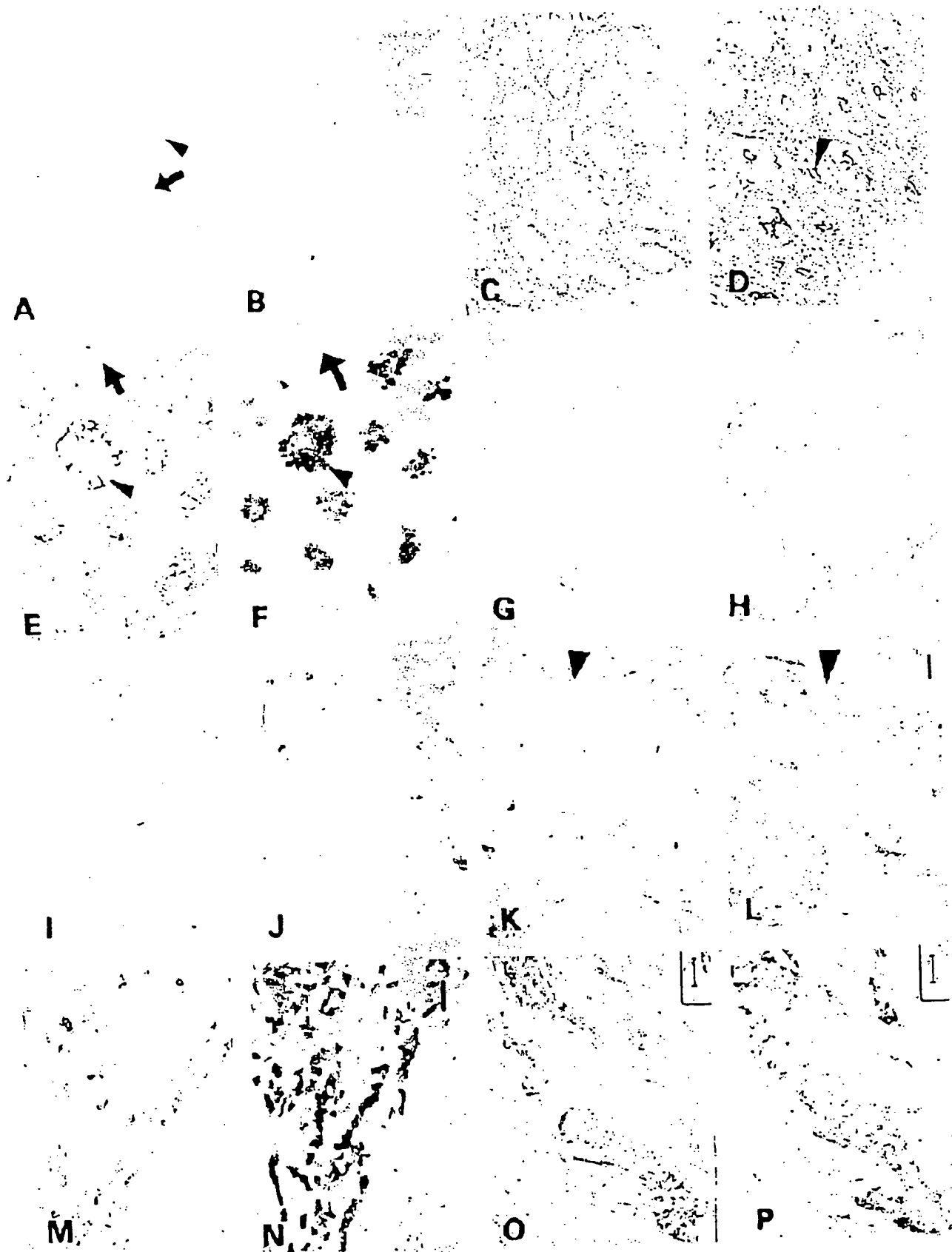
Expression of MUC1 mRNA in Normal Gastrointestinal Epithelium

By in situ hybridization, MUC1 mRNA was found to be expressed in surface epithelial cells and in gastric glands of the stomach. In all of two ileum and four colon specimens, the DIG-labeled MUC1 anti-sense RNA probe intensely stained epithelial cells, columnar cells and goblet cells (Figure 1A). The staining corresponded to the cytoplasm of columnar cells and to the perinuclear region of goblet cells. DIG-labeled sense probes did not hybridize to any tissue structure (Figure 1B). The other control experiments also showed no staining.

Expression of Apomucin Along the Normal Gastrointestinal Tract Before and After Partial Deglycosylation

The staining patterns with MUC1 mAbs before and after partial deglycosylation are shown in Figures 1 and 2. Analysis of the stained sections revealed that A76-A/C7 and HMFG-2 did not stain, as expected.

1550



MUC1 and MUC3 in Colon and Breast Tissues

any of the following untreated normal tissue sections: two stomach specimens from secretor individuals, two ileum samples from nonsecretor individuals, and 19 colon specimens (including two from ascending colon) from nonsecretor individuals (Figure 1C). SM3 behaved identically except for weak staining of the supranuclear region of columnar cells and goblet cells in one case from colon. In contrast to this, MUC1 MABs strongly labeled all normal gastrointestinal epithelia after periodate treatment, but in most cases their secretions remained unstained. Enzymatic pretreatment with α -fucosidase led to MUC1 staining exclusively in sections of the two stomach specimens, which were both secretors. Neuraminidase was without effect in all normal gastrointestinal tissues examined. Staining patterns after periodate oxidation varied with the antibody employed. A76-A/C7 staining was predominantly distributed at the apical membranes of surface mucous cells of the stomach and of columnar cells and goblet cells of the intestine (Figure 1D), whereas HMFG-2 and SM3 staining was localized in the supranuclear cytoplasm of surface mucous cells of the stomach and in the perinuclear region of columnar cells and goblet cells of the intestine. All MUC1 MABs diffusely stained gastric glands proper and pyloric glands.

MUC3 positivity in untreated normal tissue sections from the stomach was restricted to occasional cytoplasmic staining of the surface epithelium of stomach and gastric glands. In normal ileum and colon, M3.2 stained the supranuclear cytoplasm of goblet cells and columnar cells, but not secretions (Figure 1E). After periodate oxidation, mucous cells, gastric glands proper, and pyloric glands, as well as secretions in the stomach became positive. Vacuoles of goblet cells and secretions in the ileum and colon were also positive (Figure 1F).

Expression of Apomucin in Colorectal Adenomas and Carcinomas Before and After Partial Deglycosylation

The results of mucin staining in adenomas and carcinomas with and without partial deglycosylation are

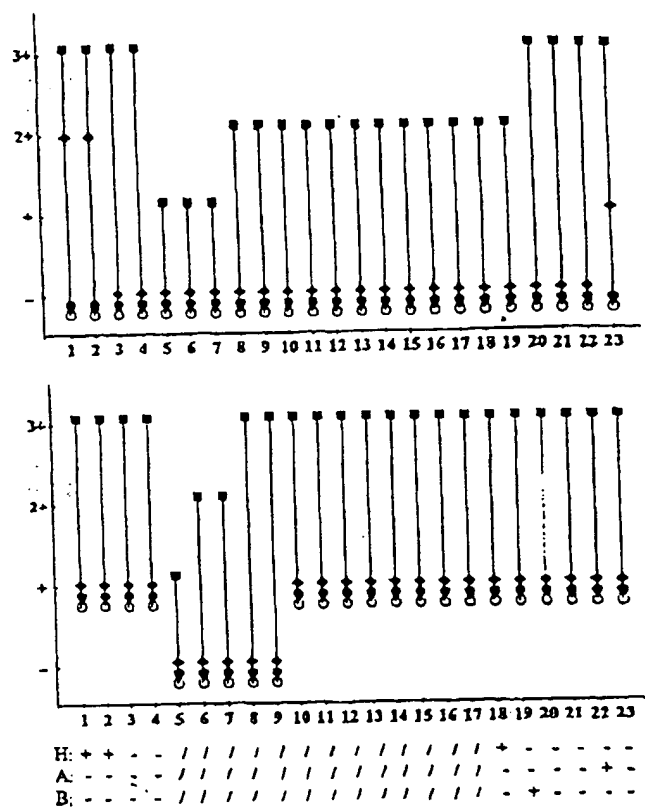


Figure 2 Comparison of the reactivity of anti-MUC1 (upper panel) and anti-MUC3 (lower panel) MABs in normal gastrointestinal tissues with or without pretreatment as indicated below, and expression of blood group antigens in epithelial cells of the same tissues (shown below the graph). -, all crypts negative; +, <30% positive crypts; 2+, 30-60% positive crypts; 3+, >60% positive crypts; O, untreated; ●, neuraminidase; ◆, α -fucosidase; ■, periodate oxidation; /, not done. Numbers 1-23 indicate individual patients: 1 and 2, stomach; 3 and 4, ileum; 5-23, colon.

shown in Figures 1, 3, and 4. For colorectal adenomas and carcinomas, similar results were obtained with all three anti-MUC1 MABs, A76-A/C7, HMFG-2, and SM3. In Figures 3 and 4, data for A76-A/C7 are shown. The anti-MUC1 MABs did not stain mildly or moderately dysplastic adenomas, with the exception

Figure 1 (A,B) Normal colon, in situ hybridization. (A) Digoxigenin (DIG)-labeled MUC1 mRNA anti-sense probe intensely stains both columnar and goblet cells; staining corresponds to the cytoplasm of columnar cells (arrowhead) and the perinuclear region of goblet cells (arrow). (B) Control section hybridized with DIG-labeled sense probe. (C,D) Normal colon. MAB A76-A/C7 (anti-MUC1) does not stain normal colon (C). After periodate treatment, it preferentially stains the apical membranes (arrowhead) of columnar and goblet cells (D). (E,F). Normal colon. MAB M3.2 (anti-MUC3) stains normal colon before (E) and after (F) periodate oxidation, although the goblet cells' vacuoles (arrowhead) and secretions (arrow) become positive only after the treatment. (G,H) Serial sections of a colon tubulovillous adenoma with severe dysplasia. MAB A76-A/C7 (anti-MUC1) does not or only weakly stains untreated adenoma cells (G) but stains tumor cells after periodate treatment (H). (I,J) Serial sections of a colon tubular adenoma with moderate dysplasia. MAB M3.2 (anti-MUC3) reacts with untreated tumor cells (I) but more strongly after periodate oxidation (J). (K,L) Serial sections of a mucinous carcinoma of the colon. M3.2 (anti-MUC3). Mucous staining (arrowhead), although present in untreated sections (K), increases after periodate treatment (L). (M,N) Serial sections of a mammary carcinoma stained with A76-A/C7 (anti-MUC1). Tumor cells are stained without pretreatment (M) but stain more strongly after periodate oxidation (N). (O,P) Serial sections of a mammary carcinoma without (O) and with periodate pretreatment (P). In both cases, tumor cells are stained with M3.2 (anti-MUC3). Bars: A-F, I, L, M-P = 50 μ m; G, H, K, L = 100 μ m.

1552

Cao, Blohm, Ghaemi, Stosiek, Xing, Karsten

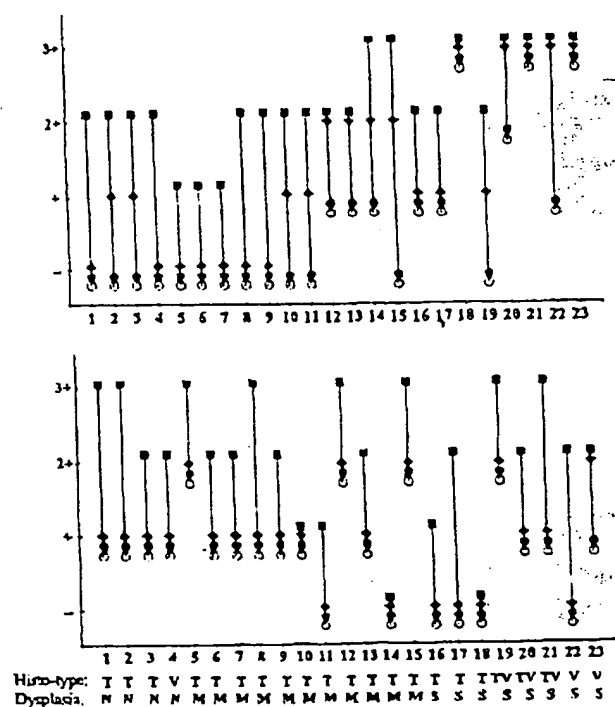


Figure 3 Comparison of the reactivity of anti-MUC1 (upper panel) and anti-MUC3 (lower panel) MABs in colorectal adenomas with or without pretreatment. —, all cells negative; +, <30% positive cells; 2+, 30–60% positive cells; 3+, >60% positive cells; O, untreated; ●, neuraminidase; ◆, α-fucosidase; ■, periodate oxidation; T, tubular adenoma; TV, tubulovillous adenoma; V, villous adenoma; N, mild dysplasia; M, moderate dysplasia; S, severe dysplasia. Numbers 1–23 indicate individual patients.

of a few weak reactions. As expected, staining was greatly enhanced in all cases after periodate treatment. These MABs, however, reacted with untreated sections of severely dysplastic adenomas; this was enhanced to a certain degree after periodate treatment (Figures 1G and 1H). Adenocarcinomas were generally reactive, but periodate treatment still slightly enhanced the reactivity. After α-fucosidase treatment slightly increased staining could be seen. In most cases, secretions in benign lesions did not react with MUC1 MABs, even after periodate treatment. In contrast, secretions of some carcinomas showed variable (from slight to moderate) reactivity with MUC1 MABs, and this was increased after periodate treatment. Neuraminidase treatment did not lead to significantly increased staining of MUC1 in gastrointestinal tumor cells and secretions.

Staining of MUC3 apomucin in colorectal benign and malignant lesions and secretions was significantly enhanced after periodate treatment (Figures 1I and 1J). A slightly increased reaction of MUC3 mAb with several severely dysplastic adenomas and carcinomas was observed after α-fucosidase treatment. Neuraminidase

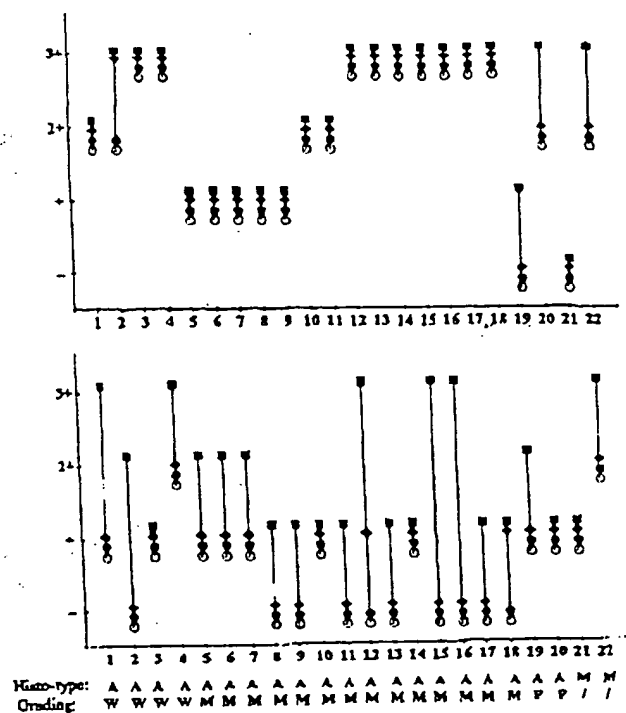


Figure 4 Comparison of the reactivity of anti-MUC1 (upper panel) and anti-MUC3 (lower panel) MABs in colorectal carcinomas with or without pretreatment. —, all cells negative; +, <30% positive cells; 2+, 30–60% positive cells; 3+, >60% positive cells; O, untreated; ●, neuraminidase; ◆, α-fucosidase; ■, periodate oxidation; A, adenocarcinoma; M, mucinous adenocarcinoma; W, well-differentiated adenocarcinoma; M, moderately differentiated adenocarcinoma; P, poorly differentiated adenocarcinoma; I, not done. Numbers 1–22 indicate individual patients.

dase treatment did not enhance staining of MUC3 in gastrointestinal tumor cells and secretions.

In adenomas with mild or moderate dysplasia, MUC1 and MUC3 (native and deglycosylated) were localized in the supranuclear cytoplasm, at the apical membrane, and/or in the apical cytoplasm of tumor cells (Figures 1E, 1F, 1I, and 1J). In carcinomas and adenomas with severe dysplasia, both mucins were observed either diffusely distributed in the whole cell or at the basolateral membrane.

Figure 5 provides a somewhat simplified overview of the staining results during the stages of colorectal carcinogenesis.

Expression of Apomucin in Normal Mammary Tissues Before and After Partial Deglycosylation

Normal mammary gland sections were reacted with A76-A/C7 and HMFG-2 before and after partial deglycosylation (data not shown). Staining was observed in all cases; it was restricted to the apical membranes of epithelial cells. Antibody SM3 did not react with normal mammary gland tissues (independent of whether

MUC1 and MUC3 in Colon and Breast Tissues

Figure 5 Comparison of staining results obtained with anti-MUC1 and anti-MUC3 antibodies on various colorectal tissues. Untreat, untreated; PO, periodate oxidation; N'ase, neuraminidase incubation; F'ase, α -fucosidase incubation. Scoring: -, no staining; +, weak staining; ++, moderate staining; +++, strong staining. Tendency of treatment effect in parentheses: -, staining not increased; ↑, staining slightly increased; TT, staining moderately increased; TTT, staining strongly increased. MUC1 immunohistochemistry results as obtained with MAb A76-A/C7 or HMFG-2.

	Normal mucosa	Adenomas with mild dysplasia	Adenomas with moderate dysplasia	Adenomas with severe dysplasia or carcinoma in situ	Invasive carcinomas	Mucinous carcinomas
MUC1						
Untreat.	-	-	++	+++	+++	-
PO	++ (TTT)	++ (TTT)	++ (TTT)	+++ (TT)	+++ (↑)	++ (↑)
N'ase	(-)	(-)	(=)	(-)	(-)	(-)
F'ase	(=)	+	+	+++ (↑)	+++ (↑)	(-)
MUC3						
Untreat.	-	+	+++	+++	+++	+++
PO	+++ (TTT)	+++ (TTT)	+++ (TTT)	+++ (TTT)	+++ (TT)	+++ (TT)
N'ase	(-)	(=)	(-)	(-)	(-)	(=)
F'ase	(=)	(=)	(-)	+	+	(=)

secretors or nonsecretors), and remained nonreactive after neuraminidase or α -fucosidase treatment. After periodate treatment, however, SM3 reacted with normal mammary gland tissue at the apical membranes of epithelial cells, similar to the localization seen with A76-A/C7 and HMFG-2.

All normal mammary tissues were negative for MUC3 with or without partial deglycosylation.

Expression of Apomucin in Breast Carcinomas Before and After Partial Deglycosylation

The staining results with anti-mucin MABs in mammary carcinoma sections with and without partial deglycosylation are shown in Figures 1 and 6. The stainability of MUC1 in mammary carcinoma cells was strongly increased after periodate oxidation (Figures 1M and 1N). Neuraminidase treatment of the sections also distinctly increased the reactivity of MUC1 with the respective MABs, whereas α -fucosidase slightly increased staining. Three patterns of MUC1 localization could be distinguished: (a) the antigen was present at the apical membranes of tumor cells in glandular adenocarcinomas; (b) the antigen was diffusely distributed in the cytoplasm of tumor cells in non-gland-forming areas of tumors; and (c) in some poorly differentiated carcinomas, a proportion of positive cells exhibited distinct granular cytoplasmic staining (focal cytoplasmic staining).

Unexpectedly, five of 20 mammary carcinomas expressed MUC3. Two further cases became positive after periodate oxidation, and in one case the staining increased after this treatment (Figures 1O and 1P). Native and deglycosylated MUC3 was diffusely distributed in the cytoplasm of tumor cells. Neuraminidase and α -fucosidase treatments had no influence on the reactivity (except in one case).

Expression of MUC1 and MUC3 apomucins before and after partial deglycosylation was not related to the secretor status of the patients.

Discussion

A common feature of glandular epithelial tissues is the apical (luminal) expression and secretion of mucins. MUC1 is generally assumed to be the major mucin expressed in normal breast, pancreas, stomach, and other glandular epithelia, but not or only sparsely in

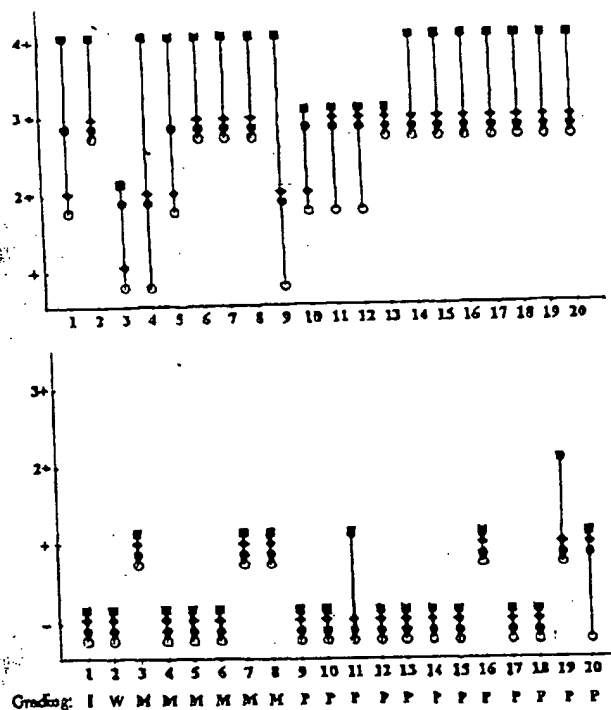


Figure 6 Comparison of the reactivity of anti-MUC1 (upper panel) and anti-MUC3 (lower panel) MABs in mammary carcinomas with or without pre-treatment as indicated below. -, all cells negative; +, <30% positive cells; 2+, 30-60% positive cells; 3+, >60% positive cells; 4+, all cells positive; O, untreated; ●, neuraminidase; ◆, α -fucosidase; ■, periodate oxidation; I, carcinoma in situ; W, well-differentiated adenocarcinoma; M, moderately differentiated adenocarcinoma; P, poorly differentiated adenocarcinoma. Numbers 1-20 indicate individual patients.

MUC1 and MUC3 in Colon and Breast Tissues

tide MUC1-specific MABs. During tumorigenesis, the apparent expression of native MUC1 showed a gradual increase from mildly through moderately to severely dysplastic adenomas. Periodate treatment greatly enhanced the reactivity of anti-MUC1 MABs with lesions of a lower grade of dysplasia, but less so with lesions of a higher grade of dysplasia. This reflects the fact that lesions of a higher grade of dysplasia express MUC1 with a lower degree of glycosylation. In malignant lesions, native MUC1 was strongly expressed, and periodate treatment only slightly enhanced the staining. This indicates that, similar to MUC1 in highly dysplastic adenomas, MUC1 in carcinomas is less glycosylated, resulting in exposed peptide epitopes that can be recognized by their corresponding antibodies. It is obvious that the grade of dysplasia of adenomas is closely related to their size. Adenomas of larger size, of a higher grade of dysplasia, or of the villous type have a higher malignant potential (Konishi and Morson 1982). The low glycosylation of MUC1 and reduced degree of glycosylation may be associated with the malignant potential and status of lesions in the colon. Several defects in glycosyltransferase activities in colorectal cancer have been described (King et al. 1994; Vavasseur et al. 1994; Yang et al. 1994), which may explain the observed changes in MUC1 glycosylation. In some carcinomas, especially mucinous carcinomas and poorly differentiated carcinomas, even periodate oxidation did not result in positive immunohistochemical staining with MUC1-specific MABs. These carcinomas apparently have lost the ability to express MUC1. Periodate oxidation therefore provides a simple method to distinguish between lack of expression and masking of epitopes by glycans. The secretions of some tumors are positive for MUC1, which is unusual because normal gastrointestinal secretions do not contain this mucin. MUC1 in tumor secretions may originate from shedding or destruction of tumor cells. MUC3 revealed a similar change in staining behavior (increased staining without pretreatment) during malignant transformation. In most adenocarcinomas, MUC3 expression was decreased compared with normal colon and benign lesions owing to downregulated synthesis (Weiss et al. 1996). Whereas normal epithelial cells and benign lesions show the typical apical distribution of MUC1 and MUC3 in addition to some supranuclear staining, malignant lesions and "benign" lesions with very high malignancy risk reveal apolar distribution of both mucins. This indicates abnormal intracellular transport in addition to incomplete glycosylation in malignant lesions. Finally, the expression of mucins within a given tumor is often heterogeneous, from zero to very high amounts per cell.

Normal breast and mammary carcinomas have been examined for the expression of MUC1 and MUC3 in a number of reports (Zotter et al. 1988;

Xing et al. 1994). In this study, changes in immunostaining in normal breast and mammary carcinomas after partial deglycosylation have been investigated. Normal mammary epithelial cells were found positive for A76-A/C7 and HMFG-2 but negative for SM3, in accord with previous reports (Burchell et al. 1987). This difference has been explained by the more extended SM3 epitope compared to the HMFG-2 epitope, which makes it more dependent on the influence of glycosylation (Burchell et al. 1989). We demonstrate here that, after periodate treatment, identical staining results were obtained with A76-A/C7, HMFG-2, and SM3. Neuraminidase or α -fucosidase treatment did not expose the SM3 epitope, indicating that sialic acid and fucose do not play major roles in epitope masking in breast epithelium.

For carcinomas of the breast, some general aspects of MUC1 immunoreactivity were found similar to that of colorectal carcinomas, e.g., decreased epitope masking compared to normal epithelial cells, disturbed polar distribution in cancer cells, and heterogeneous reactivity within a given tumor. In addition, some features of apomucin expression specific for mammary carcinomas were observed. The reactivity of MUC1 MABs with mammary carcinomas could be enhanced by prior neuraminidase treatment to a greater extent than in the case of colorectal carcinomas. This is in accord with a report by Ho et al. (1995). In another study by us, all mammary carcinomas examined were found to become positive for the MUC1-associated, normally masked Thomsen-Friedenreich glycotopope after neuraminidase treatment (unpublished data), whereas in colorectal carcinomas only 37 of 52 cases (71%) responded in this manner (Cao et al. 1995). We interpret this as indicating that in the gastrointestinal tract sialic acids contribute less to epitope masking than in the breast. In general, mammary carcinoma cells showed stronger staining of MUC1 than normal cells before or after partial deglycosylation. This may be the result of an upregulated mucin synthesis (Hilkens et al. 1995) and/or of mucin accumulation owing to abnormal transport within the tumor cells. A minority (7/20) of mammary carcinomas express ectopic MUC3. Its absence in normal epithelium was demonstrated in a previous report (Ho et al. 1993) and is confirmed in this study. Therefore, it appears that MUC3 is a neoantigen in breast cancer. We also noted that partial deglycosylation slightly enhanced its staining intensity.

The observed changes of mucin expression and its glycosylation in colorectal and mammary carcinomas may be of biological and clinical significance. For example, the exposed glycan core structures on the surface of cancer cells may lead to or enhance binding to the basal membrane and extracellular matrix (Schwartz et al. 1992), to hepatocytes (Cao et al. 1995), and to

1556

endothelial cells (Regimbald et al. 1996). Enhanced mucin expression with apolar distribution strongly reduces the contacts between epithelial cells (Hilkens et al. 1992) and may protect tumor cells from the immune system (Gimml et al. 1996). Such changes may participate in tumor progression. On the other hand, the less glycosylated, "tumor-specific" mucins in tissues, blood, and secretions may serve as neoplastic markers for clinical diagnosis and as targets for immunotherapy.

In conclusion, we have presented evidence that normal gastrointestinal epithelia express both MUC1 mRNA and the protein itself. The immunohistochemical detection of the MUC1 protein is hampered by a qualitatively different type of glycosylation compared to other secretory epithelia (e.g., breast). This can be overcome by carbohydrate-specific pretreatment with periodate, which not only leads to the detection of masked MUC1 but also allows evaluation of the changes in MUC1 glycosylation that occur in neoplastic lesions. During malignant transformation, epithelial mucins experience dramatic (yet tissue-specific) alterations in their glycosylation and cellular localization, and in some cases show ectopic expression. These changes may be significant in tumor progression, and possibly also in the clinical setting. Periodate oxidation as a complementary measure in immunohistochemistry may help us to examine and understand these changes.

Acknowledgments

Supported by Deutsche Forschungsgemeinschaft grant no. Ka 921/1.

We thank Dr D. Swallow (London) for providing the cDNA probe, and especially acknowledge the help of Ms Andrea Schaffrath (Bremen) with the *in situ* hybridization. Drs G. Pilgrim and W. Fregin are thanked for their help in providing blood group and secretor data for breast cancer patients.

Literature Cited

- Apostolopoulos V, McKenzie IFC (1994) Cellular mucins: targets for immunotherapy. *Crit Rev Immunol* 14:293-309
- Apostolopoulos V, Xing PX, McKenzie IFC (1995) Anti peptide monoclonal antibodies to intestinal mucin 3. *J Gastroenterol Hepatol* 10:555-561
- Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP (1993) Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by *in situ* hybridization. *J Histochem Cytochem* 41:1479-1485
- Bara J, Decaens C, Lordon-Rosa B, Oriol R (1992) Immunohistochemical characterization of mucin epitopes by pre-treatment of gastro-intestinal sections with periodic acid. *J Immunol Methods* 149:105-113
- Bara J, Imberty A, Pérez S, Iml K, Yachi A, Oriol R (1993) A fucose residue can mask the MUC-1 epitopes in normal and cancerous gastric mucosae. *Int J Cancer* 54:607-613
- Burchell J, Gendler S, Taylor-Papadimitriou J, Girling A, Lewis A, Mills R, Lampert D (1987) Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. *Cancer Res* 47:5476-5482
- Burchell J, Taylor-Papadimitriou J, Boshell M, Gendler S, Duhig T (1989) A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. *Int J Cancer* 44:691-696
- Carrato C, Balague C, Bolos CD, Gonzalez F, Cambus G, Planas J, Perini JM, Andreu D, Real FX (1994) Differential apomucin expression in normal and neoplastic human gastrointestinal tissues. *Gastroenterology* 107:160-172
- Cao Y, Karsten U, Hilgers J (1997a) TD-4 Workshop (Group #9): Immunohistochemical characterization of a panel of 56 antibodies with normal human small intestine, colon, and breast tissues. *Tumor Biol*. In press
- Cao Y, Karsten U, Liebrich W, Haensch W, Springer CF, Schlag P (1995) Expression of Thomsen-Friedenreich-related antigens in primary and metastatic colorectal carcinomas: a reevaluation. *Cancer* 76:1700-1708
- Cao Y, Schlag PM, Karsten U (1997b) Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and Sialosyl-Tn) in benign and malignant lesions of colon epithelium: apolar localization correspond to the malignant transformation. *Virchows Arch*. In press
- Cao Y, Stosiek P, Springer CF, Karsten U (1996) Thomsen-Friedenreich-related carbohydrate antigens in normal adult human tissues: a systematic and comparative study. *Histochem Cell Biol* 106:197-207
- Chang S-K, Dohrman AF, Eastbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS (1994) Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. *Gastroenterology* 107:28-36
- Devine PL, McKenzie IFC (1992) Mucins: structure, function, and associations with malignancy. *BioEssays* 14:619-625
- Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19:403-410
- Gimml CD, Morrison BW, Mainprice BA, Gribben JC, Boussett VA, Freeman GJ, Park-SYL, Watanabe M, Gong J, Hayes DF, Kufe DW, Nadler LM (1996) Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. *Nature Med* 2:1367-1370
- Girling A, Bartkova J, Burchell J, Gendler S, Gillett C, Taylor-Papadimitriou J (1989) A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. *Int J Cancer* 43:1072-1076
- Graham RA, Burchell JM, Taylor-Papadimitriou J (1996) The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine. *Cancer Immunol Immunother* 42:71-80
- Gum JR, Hicks JW, Swallow DM, Lagace RE, Byrd JC, Lampert DTA, Siddiki B, Kim YS (1990) Molecular cloning of CDNA's derived from a novel human intestinal mucin gene. *Biochem Biophys Res Commun* 171:407-415
- Hareven M, Tsarfay I, Zaretsky J, Kotkes P, Horev J, Zrihan S, Weiss M, Green S, Lath R, Kydar I, Wrschmer DH (1990) A transcribed gene, containing a variable number of tandem repeats, codes for a human epithelial tumor antigen. cDNA cloning, expression of the transfected gene and over-expression in breast cancer tissue. *Eur J Biochem* 189:475-486
- Hilkens J, Ligtnerberg MJL, Vos HL, Litvinov SV (1992) Cell membrane-associated mucins and their adhesion-modulating property. *Trends Biol Sci* 17:359-363
- Hilkens J, Vos HL, Wesseling J, Boer M, Storm J, van der Valk S, Calafat J, Parlarca C (1995) Is epistatin/MUC1 involved in breast cancer progression? *Cancer Lett* 90:27-33
- Ho JLL, Cheng S, Kim YS (1995) Access to peptide regions of a surface mucin (MUC1) is reduced by sialic acids. *Biochem Biophys Res Commun* 210:866-873
- Ho SB, Ewing SL, Montgomery CK, Kim YS (1996) Altered mucin

Cao, Blohm, Gimml, Stosiek, Xing, Karsten

MUC1 and MUC3 in Colon and Breast tissues

- core peptide immunoreactivity in the colon polyp-carcinoma sequence. *Oncol Res* 8:53-61
- Ho SB, Nichols GA, Lytrogg C, Yan PS, Chenwitz DL, Gum ET, Duhya R, Klin YS (1993) Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res* 53:641-651
- Jass JR, Robertson AM (1994) Colorectal mucin histochemistry in health and disease: a critical review. *Pathol Int* 44:487-504
- Jass JR, Soblin LH (1989) *Histological Typing of Intestinal Tumours*. 2nd ed. Berlin, Springer-Verlag
- Jentoft N (1990) Why are proteins O-glycosylated? *Trends Biol Sci* 15:291-294
- Karsten U, Butschak G, Cao Y, Goletz S, Hanisch F-G (1995) A new monoclonal antibody (A78-G/A7) to the Thomsen-Friedenreich pan-tumor antigen. *Hybridoma* 14:37-44
- Karsten U, Pilgrim C, Hanisch F-G, Uhlenbruck G, Kasper M, Stosiek P, Papsdorf G, Pasternak G (1988) A new monoclonal antibody (A46-B/B10) highly specific for the blood group H type 2 epitope: generation, epitope analysis, serological and histological evaluation. *Br J Cancer* 58:176-181
- King M-J, Chan A, Roe R, Warren BF, Dell A, Morris HR, Bartolo DCC, Durley P, Corfield AP (1994) Two different glycosyltransferase defects that result in GalNAc-O-peptide (Tn) expression. *Glycobiology* 4:267-279
- Konishi F, Morson BC (1982) Pathology of colorectal adenomas: a colonoscopic survey. *J Clin Pathol* 35:830-841
- Kozarsky K, Kingsley D, Krieger M (1988) Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc Natl Acad Sci USA* 85:4335-4339
- Nakamori S, Ota DM, Cleary KR, Shiotani K, Irimura T (1994) MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. *Gastroenterology* 106:53-61
- Ogata S, Uehara H, Chen A, Itzkowitz SH (1992) Mucin gene expression in colonic tissues and cell lines. *Cancer Res* 52:5971-5978
- Panton S, Gendler SJ, Spicer AP (1995) The epithelial mucin, MUC1, of milk, mammary gland and other tissues. *Biochim Biophys Acta* 1241:407-424
- Podolsky DK (1985) Oligosaccharide structures of human colonic mucin. *J Biol Chem* 260:8262-8271
- Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmermann C, Hugh JC (1996) The breast mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. *Cancer Res* 56:4244-4249
- Schaeren-Wiemers N, Gerfin-Moser A (1993) A simple protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: In situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 100:431-440
- Schwartz B, Bresalier RS, Kim YS (1992) The role of mucin in colon-cancer metastasis. *Int J Cancer* 52:60-65
- Siddiqui J, Abe M, Hayes D, Shani E, Yunis E, Kufe D (1988) Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen. *Proc Natl Acad Sci USA* 85:2320-2323
- Spiro RG (1966) Characterization of carbohydrate units of glycoproteins. *Methods Enzymol* 8:26-52
- Strous CJ, Dekker J (1992) Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* 27:57-92
- Swallow D, Gendler S, Griffiths B, Corney G, Taylor-Papadimitriou J, Bramwell ME (1987) The human tumour-associated epithelial mucins are coded by an expressed hypervariable gene locus PUM. *Nature* 328:82-84
- Taylor-Papadimitriou J, Epenetos AA (1994) Exploiting altered glycosylation patterns in cancer: progress and challenges in diagnosis and therapy. *Trends Biotechnol* 12:227-233
- Vavasseur F, Dole K, Yang J-M, Matta KL, Myerscough N, Corfield A, Parakeva C, Brockhausen I (1994) O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. *Eur J Biochem* 222:415-424
- Weiss AA, Babyatsky MW, Ogata S, Chen A, Itzkowitz SH (1995) Expression of MUC2 and MUC3 mRNA in human normal, malignant, and inflammatory intestinal tissues. *J Histochem Cytochem* 44:1161-1166
- Woodward MP, Young WW Jr, Bloodgood RA (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 78:143-153
- World Health Organization (1982) *The World Health Organization histological typing of breast tumors—second edition*. *Am J Clin Pathol* 78:806-816
- Xing PX, Apostolopoulos V, Trapani J, Prenzioska J, McKenzie IFC (1994) Peptide epitopes in breast cancer mucins. *Adv Exp Med Biol* 353:9-16
- Yang J-M, Byrd JC, Siddiki BB, Chung Y-S, Okuno M, Sowa M, Kim YS, Matta KL, Brockhausen I (1994) Alterations of O-glycan biosynthesis in human colon tissues. *Glycobiology* 4:873-884
- Zotter S, Hageman PC, Lossnitzer A, Mool WJ, Hilgers J (1988) Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Res* 48:55-101



Int. J. Cancer: 44, 969-974 (1989)
 © 1989 Alan R. Liss, Inc.



Publication of the International Union Against Cancer
 Publication de l'Union Internationale Contre le Cancer

THE SUBCELLULAR LOCALIZATION OF THE *neu* PROTEIN IN HUMAN NORMAL AND NEOPLASTIC CELLS

C.R. DE POTTER^{1,5}, J. QUACKER¹, G. MAERTENS⁴, S. VAN DAELE¹, C. PAUWELS¹, C. VERHOFFSTEDÉ², W. EECCHAUTE³ and H. ROELS¹

¹V. Goormaghtigh Institute of Pathology, ²Laboratory of Medical Microbiology and ³Laboratory of Normal and Pathological Physiology, University Hospital, Ghent; and ⁴Laboratory of Molecular Biology, State University of Ghent, Belgium.

We have examined the subcellular localization of the *neu* protein by immunohistochemistry and immuno-electron microscopy, associated with immunoblotting of normal and neoplastic tissues with 2 monoclonal antibodies (MAbs). Immuno-electron microscopy clearly reveals that *neu* protein resides only on the lateral plasma membrane of the simple epithelium of the breast and on the plasma membrane of malignant breast cells. It is also found on the membranes of the microvilli and the apical vacuoles of the cells of the proximal convoluted tubule of the kidney. In the cytoplasm, the only immunoreactivity detected with both antibodies was on the membrane of the mitochondrial cristae of normal and malignant cells. Immunoblotting reveals that the molecular weight of the membrane protein is 185 and 155 kDa for the mitochondrial protein. The cell membrane staining pattern can be revealed by light microscopic immunohistochemistry only in malignant cells and is therefore specific for malignancy. The membrane expression in normal cells cannot be visualized in this way. The mitochondrial reactivity appears as a cytoplasmic granular staining when examined under the light microscope. Similar cytoplasmic staining has been described previously in other studies with other antibodies against the *neu* protein and has lead to speculation about its function in normal and malignant cells. However, it is demonstrated in this study that it is not the known *neu*-oncogene product.

The function of the *neu* protein is unknown. Understanding its function may be facilitated by its precise localization in normal and malignant cells and by its subcellular distribution on the apical, lateral or basal part of the cell membrane. Previous studies have demonstrated that the *neu* protein is a 185 kDa membrane protein with tyrosine kinase activity (Schechter *et al.*, 1984; Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Yamamoto *et al.*, 1986). Its gene has been cloned and mapped on chromosome 17 (Coussens *et al.*, 1985; Schechter *et al.*, 1985; Fukushige *et al.*, 1986). The structure of the *neu* protein and its similarity with EGF-receptor suggest that it may be a receptor for an as yet unidentified growth factor. It is a trans-membrane protein, with a cell-external ligand binding domain and a cell-internal domain with tyrosine-kinase activity, involved in signal transduction (Coussens *et al.*, 1985). Amplification of the gene has been reported in 10-40% of breast carcinomas (Van de Vijver *et al.*, 1988a,c). It is expected to be a plasma membrane protein, but several authors have shown a cytoplasmic reactivity by light microscopy. This cytoplasmic distribution was demonstrated in kidney cells, oral mucosa, urothelium (Gullick *et al.*, 1987), normal breast tissue (Venter *et al.*, 1987; De Potter *et al.*, 1989), and breast carcinoma cells (Berger *et al.*, 1988). Cytoplasmic localization of a membrane-bound receptor could be explained by internalization of the receptor (Smith *et al.*, 1988), but it is difficult to understand why substantial immunoreactivity can be detected in cells with a barely detectable *neu* mRNA level (Kokai *et al.*, 1987).

The aim of this study was to localize this oncogenic product in normal, neoplastic and hyperplastic tissues and to look for its subcellular distribution. Therefore, an immunohistochemical investigation, and an immuno-electron microscopical study, associated with Western blot experiments, were carried out.

MATERIAL AND METHODS

Biopsy specimens

Biopsy specimens for light microscopic examination were fixed in 10% formalin for 12 hr and embedded in paraffin at 58°C using routine procedures. Five micron sections were stained with haematoxylin and eosin. The selected types of breast lesions are listed in Table I. Three hundred and thirty breast specimens from 182 patients were investigated. The peroxidase-anti-peroxidase technique was applied (De Potter *et al.*, 1989). All sections were dehydrated and mounted in Merckoglas (Merck, Darmstadt, FRG). Control sections were prepared by omitting the primary antibody. The specificity of the 3B5 antibody was tested by incubation with the primary antibody in the presence of added antigen.

For EM examination, tissues were fixed in 2% formaldehyde (from paraformaldehyde) with a 0.1 M phosphate buffer, pH 7.2. After a rinse in the same buffer, small blocks of tissue were embedded in glycolmethacrylate. Ultra-thin sections were collected on naked Ni-grids. They were floated for 30 min on TBS + 1% BSA, pH 7.6, and then kept overnight at 4°C, the primary antibody being diluted 1/100 for 3B5 and 1/3 for 9G6. The grids, which were held by forceps, were jet rinsed with TBS-BSA and floated for 1 hr on a droplet of goat anti-mouse coupled to 15 nm colloidal gold (Janssen, Bcerse, Belgium) diluted 1/20. Finally, the grids were jet-rinsed with TBS-BSA and water. The ultra-thin sections were carbon reinforced and stained for 3 min with saturated uranyl acetate in 50% alcohol and for 2 min with lead citrate.

On 6 micrographs taken from sections labelled with 9G6, the area of the ground cytoplasm and mitochondria, outside the apical zone, was measured with a Mini-mop (Kontron, Basel, Switzerland). The number of gold particles in each area was counted and the relative labelling density was calculated. The ratio of the relative labelling density of mitochondria over that of the cytoplasm—considered as background—was taken as a measure for the specific labelling of mitochondria.

Monoclonal antibodies

3B5: this MAb was raised against a synthetic peptide comprising amino acid residues 1242-1255 of the predicted sequence of the *neu* oncogene product (Van de Vijver *et al.*, 1988b). 9G6: this MAb was raised against a murine cell line, transfected with a vector, containing the human *neu* cDNA. It specifically recognizes the *neu* protein (Van de Vijver *et al.*, 1988b).

Both MAbs were tested on the SKBR-3 cell line, known to over-express the *neu* protein (Van de Vijver *et al.*, 1988b), using an immunogold silver enhancement method (Janssen). Intact cells were incubated with 3B5 and 9G6 antibodies.

⁵To whom reprint requests should be sent, at: N. Goormaghtigh Institute of Pathology, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium.

Received: May 24, 1989 and in revised form August 9, 1989.

TABLE I - RESULTS FOR THE *neu*-ONCOGENE PRODUCT IN VARIOUS ADULT BREAST TISSUES

Carcinoma patients			Non-carcinoma patients		
Lesion (number)	Results		Lesion (number)	Results	
	+	-		+	-
DCIS (6)	4	2			
Invasive duct-cell carcinoma (99)	23	76	Normal (44)	0	44
Normal in the vicinity (78)	0	78			
Normal at a distance (29)	0	29	Hyperplasia without atypia (15)	0	15
Hyperplasia without atypia (12)	0	12	Hyperplasia with atypia (7)	0	7
Hyperplasia with atypia (7)	0	7	Apocrine metaplasia (9)	0	9
Apocrine metaplasia (19)	0	19	Lactating groups (3)	0	3
Lactating groups (2)	0	2			

+: Staining pattern at the cytoplasmic membrane only is considered as being positive; -: no staining pattern at the cytoplasmic membrane.

washed with TBS-BSA, incubated with gold-labelled goat anti-mouse antibody and silver-enhanced. Only intact 9G6 stained cells showed staining of the cell membrane. A weak staining was obtained with 3B5 after permeabilization of the SKBR-3 cells with Triton X 100 (Fig. 1).

Western blot

The frozen tissue of healthy human kidneys and of positive and negative invasive ductal carcinomas of the breast was pulverized to a fine powder using a microdismembrator (Braun, Melsungen, FRG). The powder was re-dissolved and subsequently centrifuged in a Beckman ultracentrifuge at 1,500, 33,000 and 100,000 g respectively. The pellets were placed in a lysis buffer containing 1% Triton X 100 and 1 mM PMSF as protease inhibitor. Electrophoresis run on 5% polyacrylamide/SDS gels was carried out at 60 V for 16 hr. After separation, the proteins were blotted onto a nitrocellulose filter (Schleicher and Schull, Dassel, FRG, BA85; 0.45 µm) or onto a polyvinylidene difluoride microporous membrane (Millipore; 0.45 µm) for 24 hr at 50 V/0.5 A in 20% methanol containing 25 mM Tris and 192 mM glycine. The filters were incubated in a NTT buffer (150 mM NaCl-10 mM Tris.HCL, pH 8.0-0.05% Tween 20) at 4°C for 72 hr. The *neu* protein was then visualized by incubating the blot with MAb 3B5 1/3,000 or 9G6 1/100 in a NTT buffer and with a goat anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO A-5153) 1/2,000 in a NTT buffer, respectively. After each incubation the blot was washed twice in 200 ml of NTT buffer for 15 min. An additional wash was carried out in a 100 mM Tris.HCL buffer,

pH 9.5-5 mM MgCl₂. Colour development was induced by incubating with BCIP (5-bromo-4-chloro-3-indolyl phosphate; Promega, Madison, WI) and NBT (nitro blue tetrazolium; Promega) substrates in a 5-ml alkaline phosphatase buffer until sufficient specific colour was obtained. The reaction was stopped by washing with a stop buffer (5 mM EDTA-20 mM Tris.HCL, pH 8.0). The blot was subsequently rinsed in distilled water and dried. Nine samples of positive breast carcinoma, 2 samples of negative breast carcinoma, 3 samples of kidney and 2 samples of SKBR-3 cells were investigated. Control blots were carried out by pre-incubating the 3B5 antibody with the free peptide against which it was made.

RESULTS

Immunohistochemistry

The results for the various breast lesions, stained with 3B5, are summarized in Table I. In 27 carcinomas, strong staining of the plasma membrane of tumour cells was observed (Fig. 2). Twelve cases showed the *neu*-oncogene product in all tumour cells, the other cases were focally positive. There was no correlation with differentiation of the tumour. Seventy-eight carcinomas (74%), including 2 DCIS, were negative for *neu* membrane staining. Non-malignant breast lesions, normal breast and kidney did not show staining at the plasma membrane. The same results were obtained with 9G6 but staining intensity was weaker and fewer tumour cells were positive.

Several normal cells and breast carcinoma cells showed granular cytoplasmic staining with the antibodies against *neu* (Fig. 3). Experiments in which the primary antibody (3B5 and 9G6) was omitted were consistently negative. Staining of the plasma membrane of the carcinomas and cytoplasmic staining could be completely inhibited by pre-incubating the 3B5 antibody with the synthetic peptide. Pre-incubation of 9G6 with the peptide did not result in inhibition of staining. Comparison between frozen sections and paraffin-embedded material did not reveal differences in the detection of the *neu* oncogene product.

Electron microscopy

1. Neu localization

Carcinomas. Carcinoma cells exhibiting a positive and negative plasma membrane pattern for *neu* (see above) were examined at the ultrastructural level. In cases in which well-delineated cell border staining was seen, heavy colloidal gold labelling was observed over the plasma membrane with both antibodies (Fig. 4). There was no staining at the apical membrane of the cells. These cells also had prominent plasma membrane extensions. The reactivity observed by light microscopy is clearly the product of labelling intensity and plasma membrane length. In some of the cases presumed to be negative on light microscopical observation, faint labelling could still be



FIGURE 1 - (a) SKBR-3 cells stained for the *neu* oncogene product with 9G6. All tumour cells show expression at the plasma membrane. (b) SKBR-3 cells stained for the *neu*-oncogene product with 3B5. There is no staining of the cell membranes. Bar = 10µm.



FIGURE 2 - Invasive duct-cell carcinoma stained for the *neu*-oncogene product with 3B5. All tumour cells show distinct expression at the plasma membrane. Bar = 10µm.



FIGURE 3 - Renal tubules, stained for the *neu*-oncogene product with 3B5 and counterstained with haematoxylin. Cytoplasmic granular staining is observed in the tubular cells. Bar = 10µm.

observed at the ultrastructural level with both antibodies, but a positive signal was lacking in others.

Kidneys. Ultrastructural localization of *neu* in normal tissue was thoroughly studied in the proximal tubules of the kidney. Here a colloidal gold labelling for 3B5 and 9G6 was found over the villi of the brush border and at the membrane, lining the apical vacuoles (Fig. 5). *Neu* protein was excluded from the lysosomes.

Normal breast. A colloidal gold labelling for 3B5, which gave a stronger reaction than 9G6, was seen at the lateral cytoplasmic membrane of the simple epithelia of the ductuli and acini (Fig. 6).

2. Mitochondrial binding of 3B5 and 9G6

Carcinomas. The faint cytoplasmic staining observed by light microscopy with 3B5 in many cells appeared to be due to the binding of antibody to the inner mitochondrial membrane. With 9G6 no measurable binding to mitochondria could be observed in breast carcinoma cells.

Kidneys. In the proximal tubule cells and the cells of the ascending loop of Henle, the mitochondrial cristae were labelled with 3B5 and 9G6 (Fig. 7). In the loop of Henle no other reactivity was observed. With 9G6, labelling was considerably lower, but it was still on average 4.96 (± 1.29 standard error of the mean) times above the labelling of the surrounding cytoplasm in areas devoid of apical vacuoles.

Normal breast. The mitochondrial cristae were also labelled with 3B5 and 9G6. The number of mitochondria was considerably lower in these tissues than in the renal tubules.

Western blot

In pellets from positive carcinomas and from kidneys obtained at 33,000 and at 1,500 g, a faint staining band was visualized at 185 kDa with both antibodies (Fig. 8). In a 5% SDS-PAGE gels a distinct band was seen at the front. When gradient gels were used, this band gave rise to smaller bands corresponding to molecular weights below 30 kDa. These proteins may be considered as being degradation products due to proteolysis. In the *neu*-negative carcinomas, no reaction could be detected at 185 kDa, but a faint band at 155 kDa from 33,000 g pellets was observed (Fig. 9). This band was also obtained with both antibodies in some positive carcinomas and in some kidney samples that exhibited positive mitochondria. It was therefore considered as originating from mitochondria. It was not detected in tumour samples that did not show cytoplasmic staining with immunohistochemistry and containing mitochondria only in small amounts. The supernatant of all tissues at 100,000 g, corresponding to the cytoplasmic fraction, did not show a band at 185 kDa. Pre-incubation of the primary antibody with its antigenic peptide made the bands disappear at 185 and 155 kDa and in the front.

DISCUSSION

The precise localization of the *neu* protein may not only contribute to the understanding of its function, but may also provide information on the cellular orientation of the ligand binding sites. Our ultrastructural study shows an accumulation of *neu* protein at the plasma membrane of carcinoma cells that have membrane labelling by light microscopy. Electron micro-



FIGURE 4 - Electron microscopy of carcinoma cells with 3B5 and counterstained with 1% phosphotungstic acid in 1% HCL, revealing both tangentially and cross-cut segments of the plasma membrane. The plasma membrane loops are constantly labelled with colloidal gold particles between neighbouring cells. Bar = 0.25µm.



FIGURE 5 - Electron microscopy of the brush border and apical vacuoles in the proximal tubular cells of the kidney after labelling with 3B5. Colloidal gold particles decorate the villi (upper right) and gold particles can also be observed over the membranes bordering the apical pits and vacuoles (arrowhead). Bar = 0.25µm.

scopical examination also reveals labelling of the lateral membrane of the normal cuboidal epithelium of the ducti and acini of the breast. The Western blot experiments indicate that this protein is the known *neu*-oncogene product, with a molecular weight of 185 kDa. This distribution fits with a putative receptor function, where the ligand is provided by the neighbouring epithelial cells, as could be expected in a paracrine regulatory system. The localization on the cell membrane can be visualized only in malignant cells using immunohistochemistry and light microscopy and at that level it is specific for carcinomas of the breast. This staining occurs in about 26% of invasive breast carcinomas in a series of 182 patients and is not seen in normal and hyperplastic tissues (Gusterson *et al.*, 1988a,b; De Potter *et al.*, 1989).

As for normal breast tissue, immunohistochemical investigation of the kidney did not show any membrane reactivity, although the kidney contains large amounts of *neu* mRNA (Kokai *et al.*, 1987). However, EM demonstrates labelling of the membranes of the microvilli and the apical vacuoles of the proximal convoluted tubules, which confirms previous results (Mori *et al.*, 1987). This expression was confirmed by immunoblotting, in which we also detected a 185 kDa protein. The distribution of the *neu* protein on the microvilli and apical vesicles of the renal tubular cells also fits with a receptor function but with a polarity distinct from that of the breast epithelium. In this instance the ligand should be searched for in the primary urine. The above observations localize the *neu*-oncogene product at the plasma membrane of simple epithelia. Yet several authors have also localized *neu* protein in the cytoplasm, using immunohistochemical studies (Gullick *et al.*, 1987; Venter *et al.*, 1987; Berger *et al.*, 1988; De Potter *et al.*, 1989). The cytoplasmic reactivity of antibodies in foetal rat cells has even led to the conclusion that the *neu* protein plays

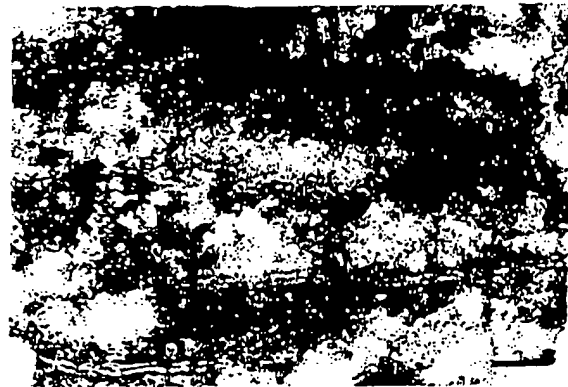


FIGURE 6 - In normal breast cells only a few gold particles were detected over adjoining plasma membranes after electron microscopical labelling with 3B5. Bar = 0.25µm.

an important role in the growth and development of a variety of tissues (Kokai *et al.*, 1987).

We also observed a positive reaction in the cytoplasm of normal renal cells and of some breast carcinoma cells by light microscopy. In EM, this staining pattern appeared to be due solely to binding of antibodies to the membranes of the mitochondrial cristae. With immunoblotting, 2 bands of respectively 185 and 155 kDa were visualized in the kidney samples. The second band was considered to be of mitochondrial origin, as it was the only band detected in negative carcinomas. Immunoelectron microscopy showing mitochondrial labelling. This mitochondrial protein is probably a cross-reacting protein, detected with both 3B5 and 9C6. It may also have

Neu PROTEIN LOCALIZATION IN HUMAN CELLS

973

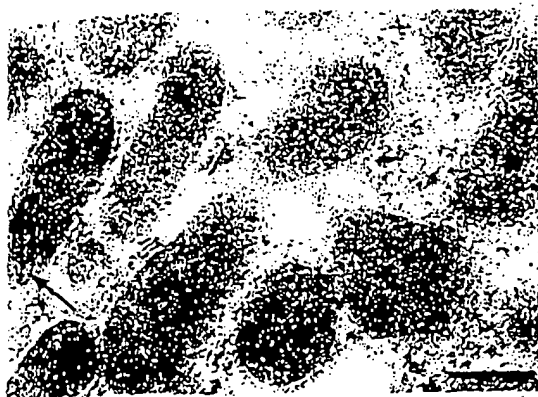


FIGURE 7 - Electron microscopy of mitochondria from proximal tubular cells, treated with 3B5. The mitochondria are heavily labelled with colloidal gold. In places (arrow) it can be recognized that the reactivity is on the mitochondrial cristae. Bar = 0.25 μ m.

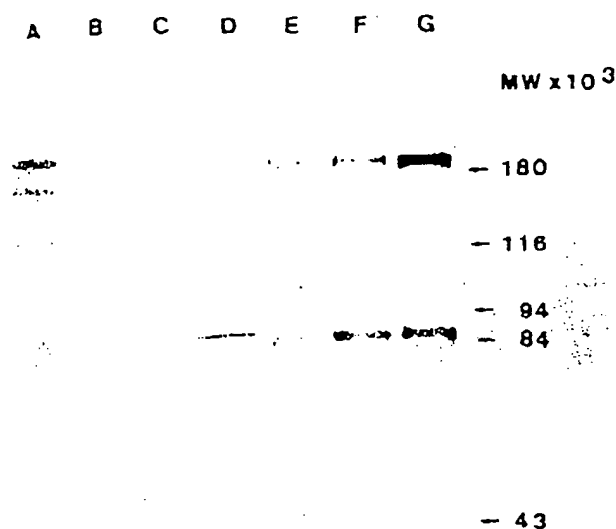


FIGURE 8 - Western blot of renal cortex and of a positive carcinoma with 3B5 on polyvinylidene difluoride filter. Lane A: Faint band at 185 and at 155 kDa disappearing at a decreasing concentration of the homogenate of the kidney in lanes B and C. Lane D: The supernatant of the kidney (A: 100 μ g was loaded in 30 μ l; B: 50 μ g in 30 μ l; C: 25 μ g in 30 μ l; D: 50 μ g in 30 μ l). Lanes E, F, and G: Band at 185 kDa for increasing concentration of the homogenate of a positive carcinoma (E: 25 μ g was loaded in 30 μ l; F: 50 μ g in 30 μ l; G: 100 μ g in 30 μ l). Lanes D-G: Additional band at 85 kDa.

been the case with several other antibodies against the *neu* protein (Gullick *et al.*, 1987; Kokai *et al.*, 1987; Venter *et al.*, 1987; Berger *et al.*, 1988). Therefore, the observation that the *neu* protein is localized in the cytoplasm of normal cells and the conclusions drawn therefrom should be interpreted with cau-

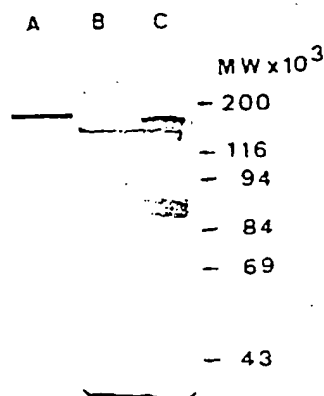


FIGURE 9 - Western blot of SKBR-3 cells and of 2 breast carcinoma specimens with 3B5. Lane A: SKBR-3 cells with a band at 185 kDa (50 μ g was loaded in 20 μ l). Lane B: Carcinoma with only cytoplasmic reactivity observed by immunohistochemistry and mitochondrial labelling observed by immuno-electron microscopy. Only a band at 155 kDa is observed (100 μ g was loaded in 30 μ l). Lane C: Carcinoma with membrane staining. Bands at 185 and at 155 kDa are observed (100 μ g was loaded in 30 μ l).

tion. A similar cross-reacting protein is detected by several monoclonal and polyclonal antibodies against CEA (Von Kleist *et al.*, 1972). The "poly-specificity" of MAbs is well known in immunohistochemistry (Andres *et al.*, 1988). Yet it is tempting to hypothesize that the mitochondrial protein might also be coded by the *neu* oncogene, but that it has a different molecular weight and a different destination in the cell. This polymorphism may be explained by post-translational processing of nascent proteins or at the mRNA level by alternative splicing (Breitbart *et al.*, 1987) or the action of different cap sites. Ultrastructural detection of the mitochondrial protein by both antibodies favours this hypothesis. Homology in the external (9G6) and in the internal (3B5) domains, as can be derived from the experiment on the SKBR-3 cell line, means that considerable similarity exists between the mitochondrial and the *neu* proteins.

From our results, we concluded that in normal simple epithelia, *neu* protein resides on the lateral plasma membrane or on the membranes of microvilli and apical vacuoles. This provides an example of 2 distinct cellular localizations of the same receptor function in both instances. Immunoreactivity with a protein other than the *neu* protein was consistently present on the inner-mitochondrial membranes, resulting in cytoplasmic staining observed by immunohistochemistry. This finding should be taken into account when interpreting *neu* oncogene over-expression in tumours for diagnostic or prognostic purposes.

ACKNOWLEDGEMENTS

We thank Dr. M. Van de Vijver and Dr. R. Nusse (Antoni Van Leeuwenhoekhuis, Amsterdam) for kindly providing the MAbs. Dr. De Potter was supported by a grant from the Nationaal Fonds voor Geneeskundig Wetenschappelijk Onderzoek.

REFERENCES

- ANDRES, G., YUZAWA, Y. and CAVALOT, F., Recent progress in renal immunopathology. *Human Pathol.*, 19, 1132-1143 (1988).
- BARGMANN, C.I., HUNG, M.-C. and WEINBERG, R.A., The *neu* oncogene encodes an epidermal-growth-factor-receptor-related protein. *Nature (Lond.)*, 319, 226-230 (1986).
- BERGER, M.S., LOCHER, G.W., SAURER, S., GULLICK, W.J., WATERFIELD, M.D., GRONER, B. and HYNES, N.E., Correlation of *c-erbB-2* gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res.*, 48, 1238-1243 (1988).
- BREITBART, R.E., ANDREADIS, A. and NADAL-GINARD, B., Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. *Ann. Rev. Biochem.*, 56, 467-495 (1987).
- COUSSENS, L., YANG-FENG, T.L., LIAO, Y.-C., CHEN, E., GRAY, A., McGRATH, J., SEEBURG, P.H., LIRKEMANN, T.A., SCHLESSINGER, J.,

- FRANCKE, U., LEVINSON, A. and ULLRICH, A., Tyrosine kinase receptor with extensive homology to EGF-receptor shares chromosomal location with *neu* oncogene. *Science*, 230, 1132-1139 (1985).
- DE POTTER, C.R., VAN DAELE, S., VAN DE VUVER, M.J., PAUWELS, C., MAERTENS, G., DE BOEVER, J., VANDEKERCKHOVE, D. and ROELS, H., The expression of the *neu*-oncogene product in normal fetal and adult human tissues. *Histopathology*, 15, 351-362 (1989).
- FUKUSHIGE, S.-I., MATSUBARA, K.-I., YOSHIDA, M., SASAKI, M., SUZUKI, T., SEMBA, K., TOYOSHIMA, K. and YAMAMOTO, T., Localization of a novel *v-erbB*-related gene, *c-erbB-2*, on human chromosome 17 and its amplification in a gastric cancer cell line. *Mol. cell. Biol.*, 6, 955-958 (1986).
- GULLICK, W.J., BERGER, M.S., BENNETT, P.L.P., ROTHBARD, J.B. and WATERFIELD, M.D., Expression of the *c-erbB-2* protein in normal and transformed cells. *Int. J. Cancer*, 40, 246-254 (1987).
- GUSTERSON, B.A., MACHIN, L.G., GULLICK, W.J., GIBBS, N.M., POWLES, T.J., ELLIOTT, C., ASHLEY, S., MONAGHAN, P. and HARRISON, S., *c-erbB-2* expression in benign and malignant breast disease. *Br. J. Cancer*, 58, 453-457 (1988a).
- GUSTERSON, B.A., MACHIN, L.G., GULLICK, W.J., GIBBS, N.M., POWLES, T.J., PRICE, P. and MCKINNA, A., Immunohistochemical distribution of *c-erbB-2* in infiltrating and *in situ* breast cancer. *Int. J. Cancer*, 42, 842-845 (1988b).
- KOKAI, Y., COHEN, J.A., DREBIN, J.A. and GREENE, M.I., Stage- and tissue-specific expression of the *neu* oncogene in rat development. *Proc. nat. Acad. Sci. (Wash.)*, 84, 8498-8501 (1987).
- MORI, S., AKIYAMA, T., MORISHITA, Y., SHIMIZU, S., SAKAI, K., SUBOH, K., TOYOSHIMA, K. and YAMAMOTO, T., Light and electron microscopical demonstration of *c-erbB2*-gene-product-like immunoreactivity in human malignant tumors. *Virchows Arch. B*, 54, 8-15 (1987).
- SCHECHESTER, A.L., STERN, D.F., VAIDYANATHAN, L., DECKER, S.J., DREBIN, J.A., GREENE, M.I. and WEINBERG, R.A., The *neu* oncogene: an *erbB*-related gene encoding a 185,000-Mr tumour antigen. *Nature (Lond.)*, 312, 513-516 (1984).
- SCHECHESTER, A.L., HUNG, M.-C., VAIDYANATHAN, L., WEINBERG, R.A., YANG-FENG, T.A., FRANCKE, U., ULLRICH, A. and COUSSENS, L., The *neu* gene: an *erbB*-homologous gene distinct from and unlinked to the gene encoding the EGF-receptor. *Science*, 229, 976-978 (1985).
- SMITH, R.M. and JARRETT, L., Receptor-mediated endocytosis and intracellular processing of insulin: ultrastructural and biochemical evidence for cell-specific heterogeneity and distinction from nonhormonal ligands. *Lab. Invest.*, 58, 613-629 (1988).
- VAN DE VUVER, M., VAN DE BERSSELAAR, R., DEVILEE, P., CORNELISSE, C., PETERSE, J. and NUSSE, R., Amplification of the *neu* (*c-erbB-2*) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked *c-erbA* oncogene. *Mol. cell. Biol.*, 7, 2019-2023 (1988c).
- VAN DE VUVER, M.J., MOOI, W.J., WISMAN, P., PETERSE, J.L. and NUSSE, R., Immunohistochemical detection of the *neu* protein in tissue sections of human breast tumours with amplified *neu* DNA. *Oncogene*, 2, 175-178 (1988a).
- VAN DE VUVER, M.J., PETERSE, J.L., MOOI, W.J., WISMAN, P., LOMANS, J., DALESIO, O. and NUSSE, R., *Neu* protein overexpression in breast cancer. Association with comedotype ductal carcinoma *in situ* and limited prognostic value in stage II breast cancer. *New Engl. J. Med.*, 319, 1239-1245 (1988b).
- VENTER, D.J., KUMAR, S., TUZI, N.L. and GULLICK, W.J., Overexpression of the *c-erbB-2* oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. *Lancet*, II, 69-71 (1987).
- VON KLEIST, S., CHAVANEL, G. and BURTIN, P., Identification of an antigen from normal human tissue that crossreacts with the carcinoembryonic-antigen. *Proc. nat. Acad. Sci. (Wash.)*, 69, 2492-2494 (1972).
- YAMAMOTO, T., IKAWA, S., AKIYAMA, T., SEMBA, K., NOMURA, N., MIYAJIMA, N., SAITO, T. and TOYOSHIMA, K., Similarity of protein encoded by the human *c-erbB-2* gene to epidermal growth factor receptor. *Nature (Lond.)*, 319, 230-234 (1986).



HER2 Assessment by Immunohistochemical Analysis and Fluorescence In Situ Hybridization

Comparison of HercepTest and PathVysion Commercial Assays

Stanley R. McCormick, MD,¹ Tamera J. Lillemoe, MD,² Janet Beneke, MD,¹
John Schrauch, MT(ASCP),¹ and John Reinartz, MD¹

Key Words: HER2; c-erbB-2; Breast cancer; Immunohistochemistry; Fluorescence in situ hybridization

Abstract

We determined HER2 protein overexpression by immunohistochemical analysis and HER2 gene amplification by fluorescence in situ hybridization (FISH) in 215 formalin-fixed, paraffin-embedded breast tumors. Pathologist concordance for immunohistochemical scoring, and HER2 status concordance, as determined by immunohistochemistry and FISH, were high for immunohistochemical 3+, 1+, and 0 tumors but poor for 2+ tumors. Consensus immunohistochemical scores correlated with absolute and chromosome 17 (CEP17)-corrected HER2 gene copy number. Among HER2-nonamplified tumors, the immunohistochemical score and mean absolute chromosome 17 (CEP17) copy number were weakly correlated. Seventeen tumors were HER2-amplified using absolute HER2 gene criteria but nonamplified when corrected for chromosome 17 polysomy (8 of these were immunohistochemical 2+). Assessment of benign epithelium within the immunohistochemical slides revealed either no staining or basolateral membrane staining, suggesting normal HER2 protein expression. Twenty tumors showing similar basolateral HER2 immunostaining were all low-moderate grade, tubule-forming, and HER2-nonamplified (17) or borderline amplified (3). Additional studies relating changes in HER2 gene content due to amplification or chromosome 17 polysomy and HER2 protein expression may be helpful to pathologists who interpret HER2 immunohistochemical slides. Breast tumors scored at 2+ should be analyzed by FISH, preferably using a dual-probe FISH assay capable of distinguishing HER2 gene amplification from chromosome 17 polysomy.

The human HER2 gene (HER-2/neu, c-erbB-2) encodes 1 member of a family of 4 transmembrane tyrosine kinases (HER1-4), the prototype of which is the epidermal growth factor receptor (HER1, crbB-1) (reviewed in Yarden and Sliwkowski¹). Various stromal-derived ligands, including epidermal growth factor, epidermal growth factor-like ligands, and neuregulins bind HER1, HER3, and HER4, inducing homodimerization and heterodimerization, phosphorylation of cytoplasmic tyrosine kinase moieties, and activation of complex signaling pathways essential for cell survival, differentiation, and proliferation.¹⁻⁴ HER2, however, is an orphan receptor with no known high-affinity ligand. HER2 becomes activated by heterodimerization after direct ligand binding by HER1, HER3, or HER4. Thus, the role of HER2 in the network of membrane receptor kinases seems to be as an amplifying coreceptor for HER1, HER3, and HER4.^{2,3} A specific erbB-2 interacting protein (ERBIN) restricts the spatial distribution of the HER2 molecule to the basolateral membrane of epithelial cells.⁵ ERBIN binds HER2, but not HER1, HER3, or HER4, and may be involved in connecting HER2 to cytosolic and cytoskeletal-associated components.⁵

The HER2 gene is central to the oncogenesis and clinical behavior of 25% to 30% of human breast cancers.^{6,7} HER2 overexpression and/or gene amplification is prognostic for node-positive^{6,7} and node-negative breast cancers,⁸ predictive for some chemotherapeutic and hormonal agents, and is an indication for trastuzumab (Herceptin, Genentech, South San Francisco, CA) therapy in patients with metastatic breast carcinoma.^{9,10} The risk of cardiac toxic effects¹¹ and the specter of patients with false-positive results accruing into breast cancer clinical trials¹² have recently focused attention

on the sensitivity and specificity of clinical HER2 assays. As the level of HER2 protein overexpression and gene amplification have prognostic and predictive relevance, HER2 assays must be semiquantitative and reproducible and discriminate normal from cancer-associated HER2 protein expression or gene content.¹³⁻¹⁵

For clinical HER2 determination, tissue-based methods, such as immunohistochemical analysis and fluorescence *in situ* hybridization (FISH), have replaced whole-tissue extraction methods, such as Southern blot analysis, enzyme-linked immunosorbent assay, and polymerase chain reaction, which may require fresh tissue or suffer dilution owing to admixing of tumor and normal cells.¹⁶ Frozen section immunohistochemical analysis, a "gold-standard" method for HER2 overexpression, is impractical in the current era of early cancer detection, in which tumor size often precludes ancillary testing of fresh tissue.

Immunohistochemical analysis is an attractive method for clinical HER2 determination owing to its retrospective potential and specific targeting of tumor cells. The plethora of available antibodies, methods, and grading schemes,^{8,17} however, have made standardization impossible. Herceptest (DAKO, Carpinteria, CA), a formalin-fixed, paraffin-embedded (FFPE)-suitable commercial immunohistochemical analysis assay, gained approval from the US Food and Drug Administration (FDA) in 1998 in part for its promise of standardization.¹⁶ Subsequent reports documented low specificity of the Herceptest 2+ category compared with gene amplification assays such as FISH.^{14,18,19} Some authors even calling for elimination of the 2+ category as a criterion for trastuzumab therapy.²⁰ Others have continued to advocate immunohistochemical analysis over FISH for its lower cost and greater availability, proposing manual or digital-assisted subtraction of background HER2 immunostaining using benign epithelium as a way of improving Herceptest specificity.^{18,21,22} Benign breast epithelium was available in only 54% of the Herceptest slides in one recent series, however.²³ Finally, proposals to subtract background epithelial staining have not clearly distinguished membrane-associated immunostaining, which may represent normal HER2 expression,^{5,23,24} from cytoplasmic staining, widely regarded as nonspecific.^{6,7,16}

One postulated advantage of immunohistochemical analysis over FISH for HER2 determination is the potential to detect protein overexpression in HER2-nonamplified tumors. Such overexpression-positive/amplification-negative tumors occur in 3% to 8% of breast cancers in most series,²⁵⁻²⁷ but were as high as 29% and 31% in comparisons of the PathVysion (Vysis, Downers Grove, IL) FISH assay with the Herceptest immunohistochemical²³ assay and the Genentech clinical trials assays,²⁸ respectively. In a study of 900 breast cancers, however, Pauletti et al¹⁴ found the

immunohistochemical-FISH discrepant tumors behaved clinically as predicted by FISH. Finally, Tubbs et al²⁰ found that the immunohistochemically 2+/FISH-negative tumors contained no detectable HER2 messenger RNA (mRNA), indicating that up-regulation of HER2 gene transcription without gene amplification is unlikely. These studies indicate that the majority of immunohistochemically positive/FISH-negative tumors are false-positive immunohistochemical results.

Pauletti et al²⁵ and Kallioniemi et al²⁶ described the simultaneous enumeration of HER2 genes and chromosome 17 centromeres by FISH, defining HER2 gene amplification as the ratio of HER2 gene copies per chromosome 17 centromere. Two-color FISH is highly sensitive (96%-98%) and specific (100%) compared with Southern blot analysis. The FDA-approved FISH assay, PathVysion, also uses a dual-probe system for simultaneous enumeration of HER2 genes and chromosome 17 centromeres in FFPE breast tumors. Another FDA-approved commercial FISH assay, INFORM (Ventana Medical Systems, Tucson, AZ), defines HER2 gene amplification as a mean absolute HER2 gene copy number of more than 4 per tumor nucleus. A study by Pauletti et al¹⁴ using the PathVysion FISH system in 900 breast cancer patients found chromosome 17 correction essential for demonstration of HER2 gene amplification; others believe it is unnecessary.²⁰

We determined the HER2 status of a diverse group of primary breast cancer specimens using the Herceptest immunohistochemical and PathVysion FISH assays. The concordance between 2 experienced surgical pathologists (S.R.M., T.J.L.) in assessing immunohistochemical scores was determined. We also compared the HER2 gene and chromosome 17 (CEP17) copy numbers in relation to immunohistochemical score and attempted background subtraction using benign epithelium as has been proposed to improve the specificity of the immunohistochemical 2+ group.^{18,21} Finally, we compared 2 definitions of HER2 gene amplification: absolute HER2 copies per tumor nucleus (threshold, 4.0) and HER2 copies per chromosome 17 centromere (threshold, 2.0).

Materials and Methods

Samples

The study used paraffinized tumor blocks from 215 breast cancers submitted for HER2 analysis from the hospitals and clinics of the Allina Health System (Minneapolis, MN), a diverse group of hospitals and outlying clinics in the upper Midwest. The specimens (11 from needle core biopsies, 72 from open or lumpectomy excisions, 49 from mastectomies, 31 from metastatic lesions, and 52 from unspecified

samples) formalin, a

Immunoh

Immunofluorescence by the μ m paraffin 37°C. The graded alcian bath) for minutes. T (DAKO) used detection of tochemical toxylin for in xylene. a

Two p. 2+, or 3+ staining w Scores of 0 negative an Discrepant 2-headed m score was u

Fluorescen

FISH v with minor SpectrumOr gene locus the alpha sa of chromos sections we finized, pre thiocyanate 37°C for 1; package ins neutral buff denatured in hybridized w posthybridiz stained with (DAPI), and -20°C and ev

FISH sli to the manuf: trumGreen s Total orange recorded, as (such as cor nuclei were i and normal n

samples) had all undergone fixation in neutral buffered formalin, although the specific fixation protocols varied.

Immunohistochemical Analysis

Immunohistochemical analysis was performed as specified by the manufacturer using the HercepTest kit. Briefly, 3- μ m paraffin sections were placed in an oven overnight at 37°C. The slides were dewaxed in xylene, rehydrated in graded alcohol, incubated in citrate buffer at 95°C (in a water bath) for 20 minutes, and then washed in water for 5 minutes. The slides then were placed on an immunostainer (DAKO) using the primary polyclonal antibody and polymer detection system supplied by DAKO. Following immunohistochemical analysis staining, the slides were placed in hematoxylin for 1 minute, dehydrated in graded alcohol, cleared in xylene, and coverslipped.

Two pathologists independently scored slides as 0, 1+, 2+, or 3+ according to DAKO guidelines. Cytoplasmic staining was ignored. Only invasive tumor was scored. Scores of 0 or 1+ were regarded as immunohistochemically negative and 2+ or 3+ as immunohistochemically positive. Discrepant immunohistochemical scores were resolved at the 2-headed microscope. The consensus immunohistochemical score was used for comparison with the FISH results.

Fluorescence In Situ Hybridization

FISH was performed as specified by the manufacturer, with minor modifications. The PathVysion kit includes a SpectrumOrange-labeled DNA probe specific for the HER2 gene locus and a SpectrumGreen-labeled probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (CEP17). Briefly, 4- μ m-thick paraffin sections were cut on organosilane-coated slides, deparaffinized, pretreated in 0.2N hydrochloric acid and sodium thiocyanate as specified, then digested in protease solution at 37°C for 12 minutes (rather than 10, as specified in the package insert). After washing, the slides were fixed in neutral buffered formalin for 10 minutes, washed, dried, denatured in 70% formamide-standard saline citrate, and hybridized with the HER2/CEP17 dual-probe mixture. After posthybridization washing, the slides were dried, counterstained with 4,6-diaminido-2-phenylindole dihydrochloride (DAPI), and coverslipped. Finished slides were stored at -20°C and evaluated within 24 hours.

FISH slides were scored by 1 observer (J.S.) according to the manufacturer's guidelines. SpectrumOrange and SpectrumGreen signals were enumerated in 60 tumor nuclei. Total orange (HER2) and green (CEP17) signals were recorded, as was the HER2/CEP17 ratio. Small samples (such as core biopsies) containing fewer than 60 tumor nuclei were included if high quality, nonoverlapping tumor and normal nuclei were evident. Chromosome 17-corrected

HER2 gene amplification was defined as an HER2/CEP17 ratio of 2.0 or more (as specified by Vysis). HER2/CEP17 ratios between 2.0 and 5.0, inclusive, were designated "low-copy" HER2-amplified, while those greater than 5.0 were designated "high-copy" amplified. HER2 gene amplification using the absolute HER2 gene copy number was defined as a mean of 4.0 or more HER2 signals per tumor nucleus.²¹ Modal CEP17 counts per 60 tumor nuclei were taken as the chromosome 17 copy number for each case, with modal CEP17 counts of 1, 2, and 3 or greater indicating chromosome 17 monosomy, disomy, and polysomy, respectively.

To examine the relationship between the immunohistochemical score and the chromosome 17 copy number of HER2-nonamplified tumors, we used both the Vysis-specified HER2/CEP17 ratio of 2.0 and a ratio of 1.8 as thresholds for amplification. The logic for including the lower HER2/CEP17 cutoff of 1.8 was that whereas the 2.0 cutoff is a clinical threshold determined by response to CAF (cyclophosphamide, doxorubicin, 5-fluorouracil) chemotherapy,²⁹ in theory, the HER2/CEP17 ratio of an HER2-nonamplified tumor should be near 1.0 (as the HER2 gene resides on chromosome 17). Thus, many tumors in the HER2/CEP17 ratio interval 1.8 to 2.0 may well be low-level HER2 gene-amplified.

Statistical Methods

FISH results were compared using total HER2 and/or CEP17 signals per 60 nuclei (or normalized to 60 when fewer tumor nuclei were available) and the *t* test method for comparison of means.

Results

Of 215 tumors, 10 were excluded for analytic failure (7 FISH, 3 immunohistochemical analysis) and 7 for insufficient tumor, leaving 198 samples for dual immunohistochemical and FISH analysis.

Pathologist concordance was 78% overall and 95%, 62%, 75%, and 83% for the 3+, 2+, 1+, and 0 immunohistochemical groups, respectively. When samples were grouped as immunohistochemical positive (2+, 3+) or immunohistochemical negative (0, 1+), pathologist concordance was 92% and 96%, respectively.

As classified by manufacturer guidelines, 86 (43.4%) of 198 cases were positive for HER2 overexpression by HercepTest (immunohistochemically positive) and 67 (33.8%) of 198 were positive for HER2 gene amplification by PathVysion (FISH-positive) (Table 1 and Image 1). Thirty-four FISH-positive cases (51%) were low-copy HER2 gene-amplified, and 33 (49%) were high-copy amplified. All high-copy amplified cases were immunohistochemically

Table 1
Immunohistochemical Score and HER2/CEP17 Fluorescence
In Situ Hybridization Ratio for 198 Breast Tumors*

Immunohistochemical Score	HER2/CEP17 Ratio			Total
	<2.0 (Non-amplified)	2.0-5.0 (Low-Copy Amplified)	>5.0 (High-Copy Amplified)	
0	51	2	0	53
1+	54	5	0	59
2+	26	14	5	45
3+	0	13	28	41
Total	131	34	33	198

CEP17, chromosome 17.

*The immunohistochemical score is the consensus score by 2 pathologists according to HercepTest guidelines. The ratio is for HER2/CEP17 signals in 60 tumor nuclei according to PathVysion guidelines. For proprietary information, see the text.

positive, while low-copy amplified cases occurred among all immunohistochemical groups (Table 1). All 41 3+ immunohistochemical cases were positive by FISH. Of 131 FISH-negative cases, 26 (19.8%) were immunohistochemically positive (all 2+). Of 67 FISH-positive cases, 7 (10.4%) were immunohistochemically negative (immunohistochemical score 0, 2 cases; immunohistochemical score 1+, 5 cases). Among the FISH-positive-immunohistochemically negative tumors, the HER2/CEP17 ratio was borderline (2.04-2.14) in 5 cases and 2.32 and 4.05 in 1 case each.

Consensus immunohistochemical scores correlated with mean HER2 gene copy number, mean CEP17 number, and the HER2/CEP17 ratio (Table 2). Among 131 FISH-negative tumors, consensus immunohistochemical scores were

weakly related to mean CEP17 copy number in a trend that approached statistical significance when the FISH threshold for amplification was lowered from 2.0 to 1.8 (Table 3). Lowering the HER2/CEP17 cutoff to 1.8 removed 11 tumors from the nonamplified group as defined by the Vysis-specified clinical cutoff of 2.0.²⁹

HER2 gene amplification as defined by absolute (mean HER2 signals per nucleus of 4.0 or more) and by chromosome 17-corrected (HER2/CEP17, 2.0 or more) criteria agreed in 178 (89.9%) of 198 breast tumors (Table 4). Seventeen tumors classified as amplified by absolute criteria were nonamplified when chromosome 17-corrected. Three tumors were HER2-nonamplified by absolute criteria but were borderline amplified (HER2/CEP17 ratios of 2.02, 2.11, and 2.17) when corrected for chromosome 17.

Of 198 immunohistochemical slides, 105 (53.0%) contained sufficient benign epithelium to evaluate background immunostaining as suggested by Jacobs et al¹⁸ and Lehr et al.²¹ Of these 105 slides, 37 (35.2%) demonstrated weak to moderate epithelial-associated immunostaining that was unrelated to the respective tumor immunohistochemical score or FISH grouping (not shown). Of 26 immunohistochemically 2+/FISH-negative cases, 11 contained sufficient benign epithelium for background assessment, 4 of which demonstrated weak to moderate immunostaining. The pattern of HER2 immunostaining associated with normal epithelium was one that highlighted the basal and lateral cell membranes, while the luminal epithelial surfaces and surrounding myoepithelial cells were invariably immunonegative (Image 2). Review of all 198 tumors revealed 20 with a

similar background were low consensus in 12, 5, 2, with basal: 3 were borderline and 2

Discuss

By using section immunohistochemistry linked HER2 with aggressive breast and clinical purposes, FISH, technical cell-specific reports of HER2 led to suggest analysis for HercepTest (Genzyme) et al²⁰ called HercepTest. Citing cost-immunohistochemical al²¹ proposed ground immunospecificity. In series, however, histochemical Hoang et al²³ HercepTest in their series, th

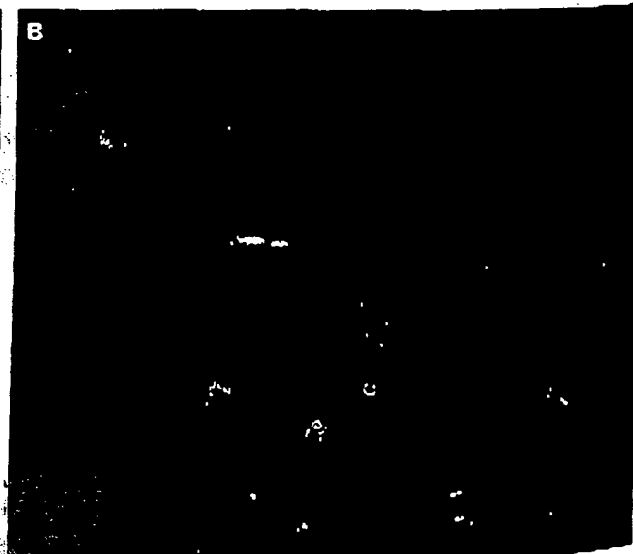
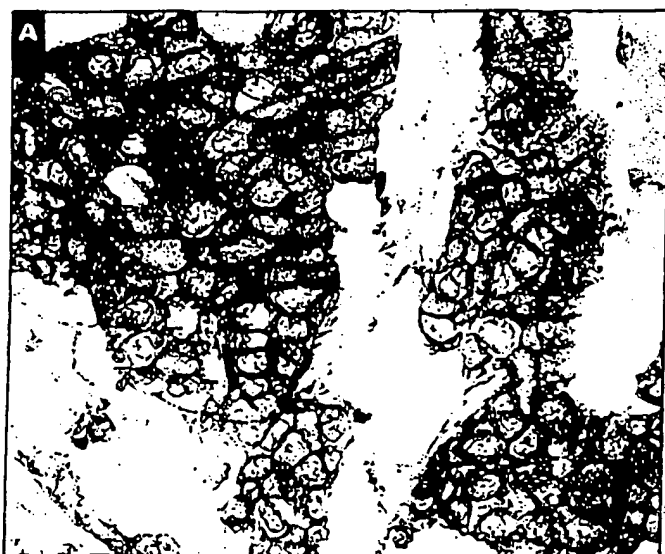


Image 1 A. Breast carcinoma, immunohistochemical score 3+, with dense, membrane-associated reaction indicating HER2 protein overexpression (HercepTest with hematoxylin counterstain, x400). B. Corresponding 2-color fluorescence in situ hybridization image showing dense clusters of SpectrumOrange (red) signal indicating HER2 gene amplification. HER2/CEP17 ratio = 11.5 (x1,000). For proprietary information, see the text.

Table 3
Immunohistochemical
Breast Tumors

Immunohistochemical Score	HER2/CEP17 cut
0	0
1+	1+
2+	2+
HER2/CEP17 cut	0
	1+
	2+

CEP17, chromosome 17. The immunohistochemical score is the consensus score by 2 pathologists according to HercepTest guidelines. The ratio is for HER2/CEP17 signals in 60 tumor nuclei according to PathVysion guidelines. For proprietary information, see the text.

similar basolateral pattern of immunostaining (Image 3): all were low to moderate grade, tubule-forming tumors with consensus immunohistochemical scores of 1+, 2+, 0, and 3+ in 12, 5, 2, and 1 tumor, respectively. Seventeen of 20 tumors with basolateral immunostaining were FISH-negative, while 3 were borderline FISH-positive (1 HER2/CEP17 ratio of 2.00 and 2 with ratios of 2.08).

Discussion

By using solid-matrix blotting methods and frozen section immunohistochemical analysis, Slamon et al^{6,7} linked HER2 protein overexpression and gene amplification with aggressive clinical behavior in a subset of primary breast and ovarian cancers. Currently, HER2 assessment for clinical purposes uses immunohistochemical analysis and FISH techniques that permit retrospective and cancer cell-specific analysis.¹⁶ Following its FDA approval in 1998, reports of HercepTest false-positive rates approaching 50%¹⁵ led to suggestions that FISH replace immunohistochemical analysis for clinical HER2 determination^{14,25} or that HercepTest 2+ breast tumors be confirmed by FISH.¹⁹ Tubbs et al²⁰ called for the FDA-mandated retraction of the HercepTest 2+ score as a criterion for trastuzumab therapy. Citing cost-effectiveness and the general availability of immunohistochemical analysis, Jacobs et al¹⁸ and Lehr et al²¹ proposed using benign epithelium to correct for background immunostaining as a way of improving HercepTest specificity. Background correction was impractical in our series, however, as only 105 (53.0%) of the tumor immunohistochemical slides included sufficient benign epithelium. Hoang et al²³ similarly reported that only 54% of their tumor HercepTest slides contained benign epithelium, although in their series, the normal epithelium appeared unstained. In our

Table 21
HER2 and CEP17 Copies by Fluorescence In Situ Hybridization vs Immunohistochemical Score in 198 Breast Tumors*

Immunohistochemical Score	Mean HER2 per Nucleus	Mean CEP17 per Nucleus	HER2/CEP17 Ratio
0	2.67	2.38	1.17
1+	2.84	2.22	1.59
2+	6.98	2.84	2.27
3+	19.65	3.15	5.23

CEP17, chromosome 17.

*Mean HER2 and CEP17 signals per nucleus in 60 tumor nuclei. The immunohistochemical score is the consensus score by 2 pathologists according to HercepTest guidelines. The ratio is for HER2/CEP17 signals in 60 tumor nuclei according to PathVysion guidelines. For proprietary information, see the text.

series, 37 of 105 immunohistochemical slides with benign epithelium showed detectable immunostaining in a delicate basal and lateral membrane pattern, with negative luminal surfaces. The basolateral pattern of immunostaining is consistent with reports of HER2 expression in benign epithelium as detected by light and electron microscopy.^{24,30} It is unclear from the reports of Jacobs et al¹⁸ and Lehr et al²¹ whether the reported background epithelial HER2 immunostaining pertains to cytoplasmic reactivity, which is generally regarded as nonspecific, or membrane-associated staining, which may represent normal HER2 protein expression.²⁴

The low level of HER2 protein associated with normal epithelium observed in our series may not have been detectable in immunohistochemical assays predating the HercepTest, which uses a potent polyclonal rabbit antibody reagent, heat-induced epitope retrieval, and polymer detection. A molecular basis for the basolateral pattern of normal HER2 immunostaining described herein is provided in a report describing a specific erbB-2 binding protein, ERBIN, which serves to localize the HER2 molecule to the basolateral membrane of mammalian epithelial cells.⁵

Table 31
Immunohistochemical Score and CEP17 Copy Number by Fluorescence In Situ Hybridization in HER2-Nonamplified Breast Tumors*

Immunohistochemical Score	Mean CEP17 per Tumor Nucleus	Comparison Grouping	P†
HER2/CEP17 cutoff = 2.0 (n = 131)			
0	2.45 (n = 51)	0 vs 1	.82
1+	2.56 (n = 54)	1 vs 2	.42
2+	3.10 (n = 26)	0 vs 2	.15
		0 + 1 vs 2	.14
HER2/CEP17 cutoff = 1.8 (n = 120)			
0	2.45 (n = 49)	0 vs 1	.82
1+	2.56 (n = 51)	1 vs 2	.23
2+	3.46 (n = 20)	0 vs 2	.05
		0 + 1 vs 2	.04

CEP17, chromosome 17.

*The immunohistochemical score is the consensus by 2 pathologists according to HercepTest guidelines. For fluorescence in situ hybridization, the mean CEP17 signal copies in 60 tumor nuclei is given. The HER2/CEP17 ratio cutoff of 2.0 is according to PathVysion guidelines (threshold, ≥2.0). The modified HER2/CEP17 ratio cutoff has a threshold of ≥1.8. The immunohistochemical group with a score of 3+ was not compared because all 3+ tumors were HER2-amplified. For proprietary information, see the text.

†t Test.

Table 4
HER2 Gene Amplification Defined by HER2/CEP17 Ratio and Mean Absolute HER2 Gene Copy Number in 198 Breast Tumors*

Absolute HER2 Signals, Mean	HER2/CEP17 Ratio		Total
	<2.0 (-)	≥2.0 (+)	
HER2 ≥4.0 (+)	17 (+/-)	63 (+/+)	80
HER2 <4.0 (-)	115 (-/-)	3 (-/+)	118
Total	132	66	198

CEP17, chromosome 17; +, positive; -, negative.

*The ratio of HER2/CEP17 signals in 60 tumor nuclei. The mean absolute is the number of HER2 signals per tumor nucleus. Parentheses show result by absolute HER2 criteria followed by result by HER2/CEP17 ratio.

Review of our immunohistochemical slides disclosed 20 low to moderate grade, tubule-forming tumors with the basal-lateral pattern of immunostaining. These 20 tumors were predominantly immunohistochemically 1+ (12 cases) or 2+ (5 cases) and HER2-nonamplified (17 cases) or borderline amplified (2 HER2/CEP17 ratios of 2.08, and 1 of 2.00). It would be of interest to know whether the discontinuous, partial membrane staining associated with the classic immunohistochemical 1+ tumor staining pattern¹⁵ reflects specific HER2 protein localization by the erbB-2 binding protein, ERBIN.

Two commercially available, FDA-approved FISH assays are available for the determination of HER2 gene amplification in FFPE breast tumors. These FISH assays



Image 21 Benign epithelium in HercepTest immunohistochemical slide (tumor not shown). Basal and lateral cell membranes are stained; while luminal surfaces (with apical snouts) and surrounding myoepithelial cells are unstained (HercepTest with hematoxylin counterstain, ×1,000). For proprietary information, see the text.

differ in whether the threshold for HER2 gene amplification is defined by absolute (as in INFORM) or chromosome 17-corrected (as in PathVysion) HER2 gene copies. Although we did not compare these 2 commercial FISH assays, by applying the absolute threshold of 4.0 or more HER2 signals per nucleus to our series, we found 17 tumors with chromosome 17 polysomy that were HER2-amplified by absolute criteria, but HER2-nonamplified when chromosome 17-corrected. Eight of these 17 chromosome 17 polysomic tumors were immunohistochemically 2+ and were regarded as immunohistochemical false-positive results. It is unclear whether the HER2 protein observed in these tumors results from the additional chromosome 17 copies (and consequent extra HER2 genes), although among all HER2-nonamplified (FISH-negative) tumors, a weak association was found between immunohistochemical score and mean CEP17 copy number that was strengthened if the HER2/CEP17 ratio threshold for amplification was lowered from 2.0 to 1.8. Finally, 3 tumors (2 with immunohistochemical scores of 2+ and 1 with a score of 1+) that were HER2-amplified by chromosome 17 correction (HER2/CEP17 ratio, 2.0 or more) contained fewer than 4.0 mean HER2 genes per tumor nucleus; all had borderline HER2/CEP17 ratios (2.02, 2.11, and 2.17). These data corroborate the use of a chromosome 17 centromeric probe in correcting for chromosome 17 copy number changes when assaying for HER2 gene amplification by FISH.

The relationship between HER2 gene copy number, surface HER2 protein density, and clinical behavior is of biologic and clinical interest.¹³⁻¹⁵ Any potential benefit from trastuzumab therapy among immunohistochemically 2+ breast tumors would likely be obscured in a clinical study as a result of the heterogeneity of HER2 gene status among the immunohistochemically 2+ group. Nevertheless, our data suggest that clinical trials using only the immunohistochemically 3+ standard to define HER2 overexpression will largely select for high-copy (>5 HER2 genes per nucleus) amplified tumors. Although exclusion of immunohistochemically 2+ tumors would eliminate many immunohistochemical false-positive results, in our series, 14 low-copy and 5 high-copy HER2-amplified tumors also would have been eliminated. The low-copy HER2-amplified tumors constitute a group that may have prognostic relevance¹⁴ and can be accurately defined only by 2-color FISH using simultaneous probes for HER2 and chromosome 17. FISH assays simultaneously enumerating HER2 and chromosome 17 copies offer the greatest resolution in detecting breast tumors with alterations of the HER2 gene. As immunohistochemical methods cannot control for low-level immunostaining that may be normal or possibly the result of chromosome 17 polysomy, and as pathologist concordance for the 2+ group is poor (62% herein), the immunohistochemically 2+ group

likely will re-
The possibi-
better with H
17-corrected
A recent clin-
chromosome
clinical outco-
mosome 17-c-
As in pr-
between FIS-
immunohisto-
intensely posi-
negative (imm-
not for the imm-
60% FISH-neg-
Of 33 tum-
FISH results, 2
FISH-negative



Image 3 **A** (Case 160), Tubular carcinoma. Reaction product lightly decorates basal and lateral membranes. Luminal surfaces are negative (HercepTest with hematoxylin counterstain, $\times 1,000$). **B** (Case 122), Moderately differentiated ductal carcinoma. Reaction product highlights basal and, focally, lateral membranes. Luminal aspects of neoplastic cells are unstained (HercepTest with hematoxylin counterstain, $\times 1,000$). **C** (Case 64), Neoplastic gland in a case of mucinous carcinoma. Reaction product highlights basal membrane, with light focal lateral membrane staining (HercepTest with hematoxylin counterstain, $\times 1,000$). For proprietary information, see the text.

likely will remain biologically and clinically heterogeneous. The possibility that absolute HER2 copy number correlates better with HER2 protein expression level than chromosome 17-corrected copy number deserves further investigation.³¹ A recent clinical study using simultaneous probes for the chromosome 17 centromere and HER2 gene found that the clinical outcome was related to absolute HER2 and to chromosome 17-corrected HER2 copy number.¹⁴

As in previous studies,^{19,23} a strong concordance between FISH (using chromosome 17 correction) and immunohistochemical analysis was found for the most intensely positive (immunohistochemical score 3+) and negative (immunohistochemical score 0 or 1+) tumors but not for the immunohistochemically 2+ group (approximately 60% FISH-negative and 40% FISH-positive herein).

Of 33 tumors with discrepant immunohistochemical and FISH results, 26 were immunohistochemically positive and FISH-negative (all immunohistochemically 2+), and only 7

were immunohistochemically negative and FISH-positive (all low-copy HER2-amplified). The clinical significance of the immunohistochemically positive-FISH-negative tumors has been addressed recently in a large cohort ($n = 900$) of breast cancers, in which no outcome difference was found between the immunohistochemically positive-FISH-negative and the immunohistochemically negative-FISH-negative tumors.¹⁴ In addition, a recent study of mRNA expression in breast tumors found the immunohistochemically 2+-FISH-negative tumors expressed no HER2 mRNA, suggesting the immunostaining of these single HER2 gene copy tumors was not the result of up-regulated HER2 gene transcription.²⁰

The clinical significance of the rare (7 of 198) immunohistochemically negative-FISH-positive breast tumors is uncertain. In our series, all immunohistochemically negative-FISH-positive tumors were low-copy (2-5 HER2 signals per CEP17 signal) HER2-amplified. Clinical studies using 2-color FISH to stratify tumors by absolute and chromosome

17-corrected HER2 copy number, with correlation to prognostic and therapeutic endpoints, should clarify the immunohistochemically negative-FISH-positive cases.

FISH and the HercepTest assay are highly concordant for immunohistochemically 3+ and negative (immunohistochemical score 0, 1+) breast tumors, although the immunohistochemically 2+ group includes both HER2-amplified and HER2-nonamplified tumors. The mechanisms of HER2 expression among nonamplified tumors with immunohistochemical 1+ and 2+ immunostaining are unclear, but may reflect intact normal pathways of HER2 expression and membrane localization or may possibly involve chromosome 17 polysomy. Detection of normal levels of HER2 protein on benign epithelium and HER2-nonamplified tumors is not surprising given potency of the HercepTest assay. ERBIN, a receptor protein specifically localizing HER2 to the basolateral membrane,⁵ may provide a molecular basis for understanding the microscopic patterns of immunostaining observed in association with benign epithelium and some tubule-forming, HER2-nonamplified breast tumors. Finally, when FISH is used to detect HER2 gene amplification, use of a chromosome 17 centromeric probe (as in the PathVision kit) is crucial for distinguishing breast tumors with low-level HER2 gene amplification from those with chromosome 17 polysomy. It seems unlikely that adjustment of the immunohistochemical score using benign epithelium will improve the specificity of the HercepTest 2+ group. Continuing advances in early breast cancer detection and the use of smaller biopsy techniques are trends that will lead to smaller biopsy specimens, further reducing the amount of additional benign epithelium available as internal control tissue.

From the ¹Department of Pathology and the United Hospital Breast Center, United Hospital, St Paul, MN; and ²Department of Pathology and the Virginia Piper Breast Center, Abbott Northwestern Hospital, Minneapolis, MN.

Funded by the Breast Cancer Clinical Action Group of the Allina Health System, Minneapolis, MN.

Address reprint requests to Dr McCormick: Dept of Pathology, United Hospital, 333 N Smith Ave, St Paul, MN 55102.

Acknowledgments. We gratefully acknowledge the expertise of Paula Overn, Donna Schwab, and Sue Knittel in preparing the histologic and immunohistologic slides.

References

- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001;2:127-137.
- Klappper LN, Glatche S, Vaisman N, et al. The erbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci U S A*. 1999;96:4995-5000.
- Graus-Porta D, Beerli R, Daly JM, et al. ErbB-2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. *EMBO J*. 1997;16:1647-1655.
- Burden S, Yarden Y. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron*. 1997;18:847-855.
- Borg J-P, Marchetto S, Le Bivic A, et al. ERBIN: a basolateral PDZ protein that interacts with the mammalian ERB2/HER2 receptor. *Nat Cell Biol*. 2000;2:407-413.
- Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177-182.
- Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-712.
- Press MF, Pike MC, Chazin VR, et al. Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res*. 1993;53:4960-4970.
- Nicholson BP. Ongoing and planned trials of hormonal therapy and trastuzumab. *Semin Oncol*. 2000;27:33-37.
- Fornier M, Esteva FJ, Seidman AD. Trastuzumab in combination with chemotherapy for the treatment of metastatic breast cancer. *Semin Oncol*. 2000;27:38-45.
- Sparano JA. Cardiac toxicity of trastuzumab (Herceptin): implications for the design of adjuvant trials. *Semin Oncol*. 2001;28:20-27.
- Nabholtz J-M, Slamon DJ. New adjuvant strategies for breast cancer: meeting the challenge of integrating chemotherapy and trastuzumab (Herceptin). *Semin Oncol*. 2001;28:1-12.
- Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. Oncologic Drugs Advisory Committee, 58th meeting [transcript]. Bethesda, MD; September 2, 1998. Available at: <http://www.fda.gov/ohrtms/dockets/ac/cder98t.htm>. Accessed June 2001.
- Pauletti G, Dandekar S, Rong H, et al. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J Clin Oncol*. 2000;18:3651-3664.
- Mass R. The role of HER-2 expression in predicting response to therapy in breast cancer. *Semin Oncol*. 2000;27:46-52.
- Ross JS, Fletcher JA. HER-2/neu (c-erb-B2) gene and protein in breast cancer. *Am J Clin Pathol*. 1999;112 (suppl 1):S53-S57.
- Press MF, Hung G, Godolphin W, et al. Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res*. 1994;54:2771-2777.
- Jacobs TW, Gown AM, Yacobi H, et al. Specificity of HercepTest in determining HER-2/neu status of breast cancer using the United States Food and Drug Administration-approved scoring system. *J Clin Oncol*. 1999;17:1983-1987.
- Lebeau A, Deimling D, Kaltz C, et al. HER-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. *J Clin Oncol*. 2001;19:354-363.
- Tubbs RR, Peitay JD, Roche FC, et al. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J Clin Oncol*. 2001;19:2714-2721.
- Lehr of HI hybrid analysis.
- Blood HER score: Postcancer Chron.
- Hoan amplification: breast.
- Yeh I-gene in 1993.
- Paulet quant breast hybrid.

21. Lehr H-A, Jacobs TW, Yaziji H, et al. Quantitative evaluation of HER-2/neu status in breast cancer by fluorescence in situ hybridization and by immunohistochemistry with image analysis. *Am J Clin Pathol*. 2001;115:814-822.
22. Bloom K, de la Torre-Bueno J, Press MF, et al. Comparison of HER-2/neu analysis using FISH and IHC when Herceptin is scored using conventional microscopy and image analysis. Poster presentation at: 23rd Annual San Antonio Breast Cancer Symposium; 2000; San Antonio, TX. Poster 416: Chroma Vision.
23. Hoang MP, Sahin AA, Ordonez NG, et al. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. *Am J Clin Pathol*. 2000;113:852-859.
24. Yeh I-T, Keykhah S, Selig AM, et al. Expression of the *erbB2* gene in benign and proliferative breast tissue. *Surg Pathol*. 1993;5:109-120.
25. Pauletti G, Godolphin W, Press MF, et al. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene*. 1996;13:63-72.
26. Kallioniemi O-P, Kallioniemi A, Kurisu W, et al. ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci U S A*. 1992;89:5321-5325.
27. Persons JO, Borelli KA, Hsu PH. Quantitation of HER-2/neu and c-myc gene amplification in breast carcinoma using fluorescence in situ hybridization. *Mod Pathol*. 1997;10:720-727.
28. Mass RD, Sanders C, Kasian C, et al. The concordance between the clinical trials assay (CTA) and fluorescence in situ hybridization (FISH) in the Herceptin pivotal trials [abstract]. *Proc Annu Meeting Am Soc Clin Oncol*. 2000;19.
29. PathVysion HER-2 DNA probe kit [package insert]. Downers Grove, IL: Vysis; 1998.
30. De Potter CR, Quatacker J, Maertens G, et al. The subcellular localization of the neu protein in human normal and neoplastic cells. *Int J Cancer*. 1989;44:969-974.
31. Scollisi J, Balazs M, Feuerstein BG, et al. ERBB-2 (HER2/neu) gene copy number, p185 overexpression and intratumor heterogeneity in human breast cancer. *Cancer Res*. 1995;55:5400-5407.